

THE ROLES OF SOX2 AND SOX3 DURING OTIC AND EPIBRANCHIAL  
DEVELOPMENT IN ZEBRAFISH

A Dissertation

by

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## ABSTRACT

Sox2 and Sox3 are SoxB1 transcription factors that act redundantly in the neural ectoderm, but also are uniquely required in other systems. To address functions of *sox2* and *sox3* in otic and epibranchial development, we generated knockouts and heat shock-inducible transgenes in zebrafish.

Expression of *sox3* is one of the earliest markers of Fgf-dependent otic/epibranchial placode induction. We found that *sox2* is also expressed in the early placode. Mutant analysis, and low-level misexpression, showed that *sox2* and *sox3* act redundantly to establish a full complement of otic/epibranchial cells. Disruption of *pax8*, another early regulator, caused similar placodal deficiencies to *sox3* mutants or *pax8-sox3* double mutants, suggesting that *sox3* and *pax8* operate in the same pathway. High-level misexpression of *sox2* or *sox3* during early stages cell-autonomously blocked placode induction, whereas misexpression several hours later could not reverse placodal differentiation. In an assay for ectopic placode-induction, partial knockdown of *sox3* significantly enhanced ectopic induction of *pax8*, whereas full knockdown of *sox3* inhibited this process. These findings show that *sox2* and *sox3* are together required for proper otic and epibranchial induction, but the level of expression must be tightly regulated to avoid suppression of differentiation and maintenance of pluripotency.

Mutant analysis at later stages of otic development showed that *sox2* and *sox3* are uniquely required for sensory development and otic neurogenesis respectively. Moderate misexpression of *sox2* during placodal stages led to development of otic vesicles with expanded sensory and reduced neurogenic domains. However, high-level misexpression of *sox2* or *sox3* expanded both sensory and neurogenic domains, filling the medial and lateral halves of the otic vesicle, respectively.

Disruption of medial factor *pax2a* eliminated the ability of *sox2/3* misexpression to expand sensory but not neurogenic domains. Additionally, mild misexpression of *fgf8* during placodal development was sufficient to specifically expand the zone of prosensory competence. Later, cross-repression between *atoh1a* and *neurog1* helps maintain the sensory-neural boundary. We also show that *sox2* and *sox3* exhibit intrinsic differences in promoting sensory vs. neural competence, but at high levels they can mimic each other to enhance both states. Regional cofactors like *pax2a* and *fgf8* also modify *sox2/3* functions.

## DEDICATION

I would like to dedicate this dissertation to my mother and father, for their unconditional love and support.

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## CONTRIBUTORS AND FUNDING SOURCES

### Contributors

This work was supervised by a dissertation committee consisting of Professor Bruce B. Riley (advisor) of the Department of Biology, Professor Arne C. Lekven of the Department of Biology, Professor L. Rene Garcia of the Department of Biology, and Professor Sarah E. Bondos of the Department of Molecular and Cellular Medicine.

The work in Chapter 2 was done in collaboration with Dr. Jinbai Guo, who is a post-doc in the Riley lab, and Kirstin Maulding, a former undergraduate student in the lab. Dr. Jinbai Guo helped in generating *sox2<sup>x50</sup>*, *sox3<sup>x52</sup>* and *sox3<sup>x53</sup>* lines shown in Figure 2.1A, Kirstin Maulding performed experiments shown in Figure 2.5, Figure 2.8A, B, D, E, G-L and Figure 2.9.

The work in Chapter 3 was done in collaboration with Dr. Shruti Vemaraju, Dr. Elly M. Sweet and Dr. Hye-Joo Kwon. Dr. Shruti Vemaraju and Dr. Elly M. Sweet are former graduate students in the Riley lab, Dr. Hye-Joo Kwon is a former post-doc in the lab. Dr. Hye-Joo Kwon performed pilot studies lead to experiments shown in Figure 3.2C-F, H and Figure 3.8A-D. Dr. Sweet performed experiment shown in Figure 3.9E, F. The model Figure 3.11 was generated by Dr. Bruce B. Riley.

All other work conducted for the dissertation was completed by the student, under the advisement of Dr. Bruce B. Riley of the Department of Biology.

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## NOMENCLATURE

bHLH	Basic helix-loop-helix, a protein structure motif
Cas9	CRISPR-associated protein 9
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
dpf	days post-fertilization
EMT	Epithelial-to-mesenchymal transition
Fgf	Fibroblast growth factor
GFP	Green fluorescent protein
<i>gsc</i>	<i>goosecoid</i>
hpf	hours post-fertilization
<i>ngn1/neurog1</i>	<i>neurogenin1</i>
MO	Morpholino oligomer
Morphants	MO-injected embryos
SAG	Statoacoustic ganglion
TALEN	Transcription activator-like effector nucleases

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# CHAPTER I

## INTRODUCTION

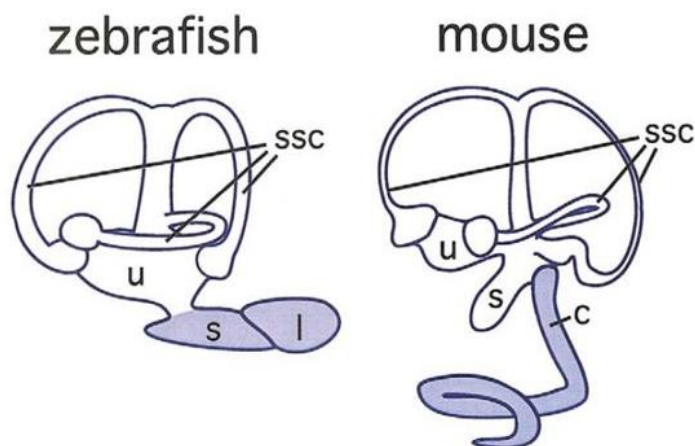
### **STRUCTURE OF INNER EAR**

The inner ear is a complex three-dimensional organ that is the basis of auditory (hearing) and vestibular (balance) functions in vertebrates. Zebrafish inner ear is structurally similar to that of other animals like birds and mammals, comprising three semicircular canals and three interconnected chambers. Zebrafish forms utricle, saccule and lagena, whereas mouse forms utricle, saccule and cochlea (Fig. 1.1). Each canal or chamber houses a patch of sensory epithelium, called cristae (in semicircular canals) or maculae (in other chambers) (Haddon and Lewis, 1996). Zebrafish cristae and utricular macula mediate vestibular function, whereas the saccular and lagenar maculae are involved in auditory function (Haddon and Lewis, 1996; Whitfield et al., 2002). In birds and mammals, auditory function is mediated by sensory epithelia in the cochlea, while cristae and maculae in the utricle and saccule contribute to vestibular function (reviewed by Riley and Phillips, 2003).

### **STRUCTURE OF SENSORY EPITHELIUM**

The basic structure of sensory epithelium is also highly conserved, comprising intermingled sensory hair cells and non-sensory support cells in a salt-and-pepper pattern (Fig. 1.2). Sensory hair cells are the mechanotransducers that detect acceleration or sound vibration

through lateral deflection of long ciliary bundles. These cilia are located at the apical surface of hair cells, protruding into the fluid-filled lumen of the inner ear. Deflection of cilia opens mechanosensitive ion channels, causing ion influx into the hair cell to stimulate neurotransmitter release to post-synaptic neurons (Fig. 1.2). These innervating neurons, known as the VIII<sup>th</sup> cranial ganglion or the statoacoustic ganglion (SAG), relay sensory signals from hair cells to higher processing centers in the brain. Support cells are important for survival of hair cells by providing trophic factors (Haddon et al., 1998; Haddon et al., 1999). Support cells also play a stem cell-like role in the inner ear, regenerating lost hair cells via proliferation or direct transdifferentiation (Millimaki et al., 2010; reviewed by Wan et al., 2013).

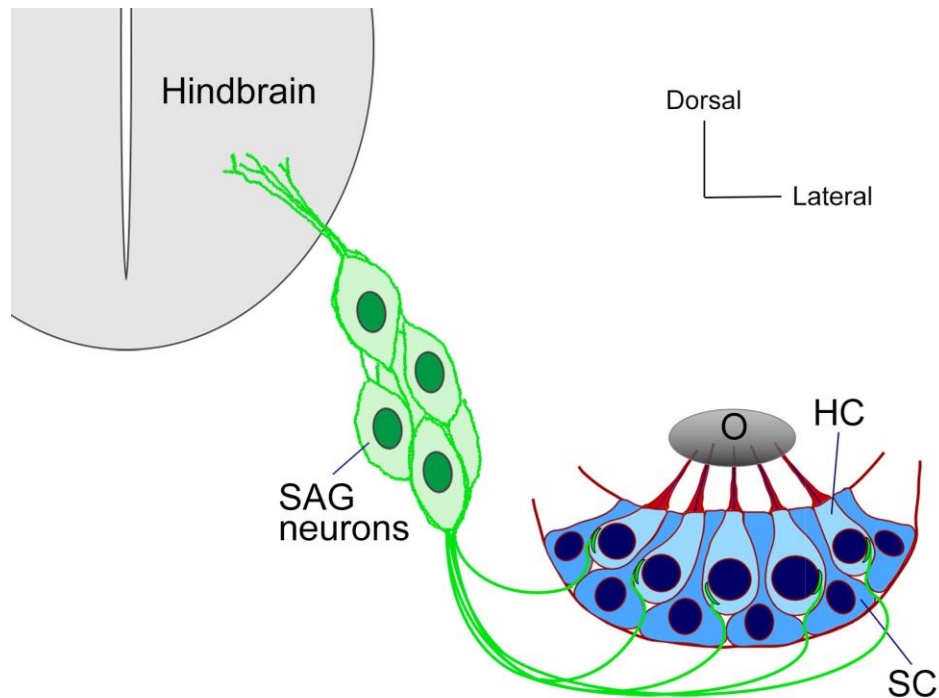


**Figure 1.1. General structure of the adult inner ear. (Reprinted with permission from Riley and Phillips, 2003)\***

Representative illustration of the adult inner ear structures in zebrafish and mouse. Blue shaded regions indicate auditory regions. Lateral views, with anterior to the left. Abbreviations: c, cochlea, l, lagena; s, saccule; ssc, semicircular canals; u, utricle.

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\* Figure 1.1 is reprinted with permission from “Ringing in the new ear: resolution of cell interactions in otic development.” by Riley, B.B., Phillips, B.T., 2003, *Developmental Biology* 261, 289-312, Copyright 2003 by Elsevier.



**Figure 1.2. Illustration of sensory epithelium and SAG neurons in the inner ear.**

Schematic cartoon showing the general structure of sensory epithelium, using utricular macula as an example, as well as neurons of the statoacoustic ganglion (SAG) that innervate hair cells to the hindbrain. HC, hair cell, SC, support cell, O, otolith.

In zebrafish, hair cells in maculae are connected to calcium carbonate aggregates called otoliths (Fig. 1.2), which help deflect hair cell cilia therefore facilitate detection of acceleration, gravity and sound. Utricular and saccular maculae are the first two sensory epithelia developed in the zebrafish inner ear, with the first mature hair cells appearing by 22 hours post-fertilization (hpf). Otoliths appear nearly simultaneously as small “seeding” particles accumulate at the tips of hair cell cilia (Riley and Grunwald, 1996; Riley et al., 1997). The utricular macula begins to function as a vestibular organ by 24 hpf, and the saccular macula begins to mediate hearing by 48 hpf (Lu and DeSmidt, 2013; Riley and Moorman, 2000). Cristae and the lagenar macula emerge by 48 hpf and 11 dpf (days post fertilization), respectively (Riley and Moorman, 2000), although

it is not clear when they begin to function. In any case, the utricular and saccular maculae provide an excellent model to study the development of sensory epithelia, due to their early development and functional integration. In addition, these maculae develop adjacent to the neurogenic domain of the inner ear, which produces neuroblasts that produce the entire SAG during a similar time window (see below). How sensory and neurogenic domains are regulated and patterned to develop in abutting domains concomitantly in zebrafish inner ear is not well understood. This will be examined and discussed in Chapter III.

## **DEVELOPMENT OF SENSORY EPITHELIUM**

Sensory hair cells and non-sensory support cells both differentiate from a common domain of progenitor cells within a classical “equivalence group” marked by expression of basic helix-loop-helix (bHLH) transcription factor *atoh1*, which is the vertebrate homolog of *Drosophila* proneural gene *atonal* (Bermingham et al., 1999; Millimaki et al., 2007). In zebrafish, there are two *atonal* homologs, *atoh1a* and *atoh1b*, which are both essential for hair cell development, but are differentially required during different developmental stages (Millimaki et al., 2007). Initially, *atoh1b* expression marks an equivalence group in the medial side of the preotic placode (marked by expression of *pax8*, see later sections in this chapter) at 10.5 hpf (right after gastrulation), which is the first sign of sensory development in zebrafish. *Atoh1b* activates Delta-Notch signaling to restrict the size of equivalence group into two domains, the utricular and saccular primordia, by 12 hpf (Millimaki et al., 2007). Through Delta-Notch mediated lateral inhibition, first two hair cells in each anlagen are specified and start to express *atoh1a* at 14 hpf (Millimaki et al., 2007). They subsequently differentiate into “tether cells” that function to initiate development of otoliths

(Riley et al., 1997). Later-forming hair cells are produced from a group of *atoh1a* expressing cells that are activated by Notch and Fgf beginning at 20 hpf via a secondary wave of prosensory induction (Millimaki et al., 2007). Starting from this stage, *atoh1a* is the predominate proneural gene functions in the otic vesicle and is required to maintain *atoh1b* expression in a subset of cells (Millimaki et al., 2007). Later-forming hair cells are specified by high level of *atoh1a*, while their neighboring cells, receiving strong Delta-Notch mediated repression, lose *atoh1* expression and adopt support cell fate. In zebrafish *mind bomb* (*mib*) mutants, lacking Delta-Notch signaling, the restriction of the equivalence group fails and results in otic vesicles with supernumerary hair cells at the expense of support cells (Haddon et al., 1999; Millimaki et al., 2007; Riley et al., 1999). How *mib* affects the patterning of sensory and neurogenic domain in the otic vesicle will be explored in Chapter III. In my studies, sensory specification was visualized by examining expression of *atoh1a*, whereas fully differentiated hair cells were visualized by expression of *pou4f3* (previously named *brn3c*), a POU-domain transcription factor and immediate downstream target of Atoh1a. Hair cells were monitored in live embryos using transgenic line *brn3c:GFP* (Xiao et al., 2005).

## NEUROGENIC DEVELOPMENT OF THE INNER EAR

Sensory information from the inner ear is transmitted to hindbrain through neurons of the VIII<sup>th</sup> cranial ganglion, also called the statoacoustic ganglion (SAG). Cell bodies of SAG neurons reside between the otic vesicle and the hindbrain, but SAG precursors (neuroblasts) originate in the otic epithelium immediately adjacent to the developing maculae. SAG neuroblasts undergo a complex developmental program involving sequential steps of specification, delamination,



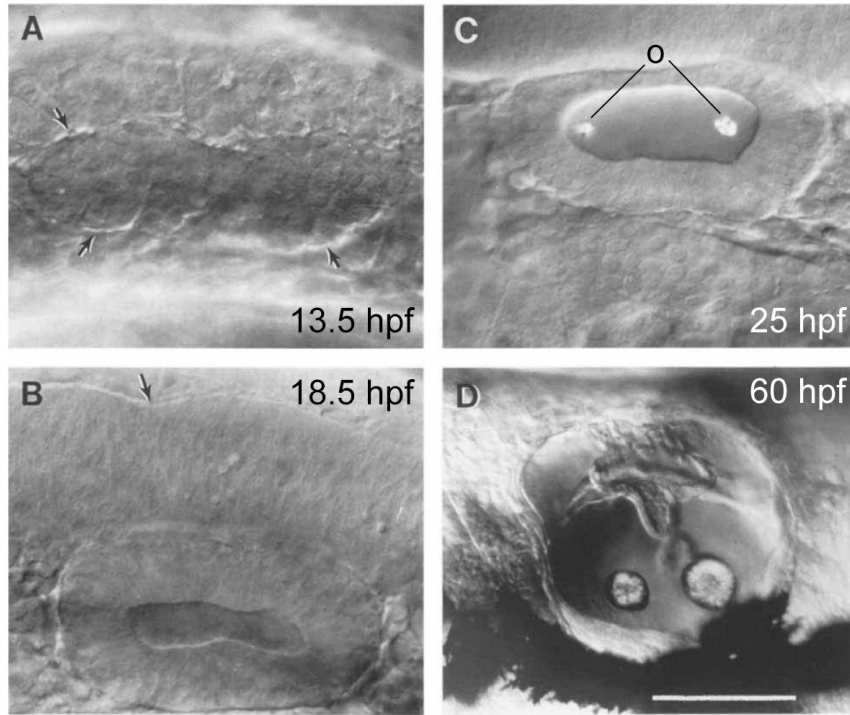
proliferative expansion and differentiation to become mature SAG neurons. First, a subset of cells in the floor of the otic vesicle adjacent to the developing sensory epithelia are specified to be SAG neuroblasts, marked by expression of *neurogenin1* (*neurog1*, or *ngn1*) (Andermann et al., 2002; Ma et al., 1998). *Neurog1*, also an *atonal*-related bHLH transcription factor, is essential for determining neuroblast fate, while also activating Delta-Notch signaling to limit neurogenesis (Abello et al., 2007; Kantarci et al., 2015; Ma et al., 1998). A subset of *neurog1*+ neuroblasts also express *goosecoid* (*gsc*), which promotes epithelial-to-mesenchymal transition (EMT) to help these precursors delaminate and leave the otic epithelium (Kantarci et al., 2016). After leaving the otic vesicle, SAG neuroblasts downregulate *neurog1* and upregulate another related bHLH factor *neurod* (Andermann et al., 2002; Korzh et al., 1998). Subsequently, SAG precursors form a pool of proliferating progenitors called the transit-amplifying pool (Camarero et al., 2003; Vemaraju et al., 2012). After migrating to a position between the otic vesicle and hindbrain, transit-amplifying cells lose *neurod* expression and upregulate neuronal markers like *islet1* (*isl-1*) as they differentiate into mature SAG neurons (Korzh et al., 1998; Vemaraju et al., 2012).

Recent studies showed that Fgf signaling plays an essential role in regulating multiple steps of SAG development. The initial specification of *neurog1*+ neuroblasts, which starts from 17 hpf and peaks around 24 hpf, requires a moderate level of Fgf from the nascent maculae and adjacent hindbrain (Vemaraju et al., 2012). Fgf is also required to induce expression of the EMT-promoting factor *gsc* in neuroblasts (Kantarci et al., 2016). Later, Fgf levels in the otic vesicle rise due to accumulation of mature SAG neurons, which express *fgf5*, and eventually exceeds an upper limit for neuroblast specification (Vemaraju et al., 2012). As a result, expression of *neurog1* in the otic vesicle naturally begins to decrease from 24 hpf and ceases completely around 42 hpf (Vemaraju et al., 2012). Rising Fgf5 in mature SAG also feeds back to the transit-amplifying pool to slow

down their differentiation, ensuring a stable pool of proliferating progenitors and steady production of mature neurons throughout development (Vemaraju et al., 2012).

## **MORPHOGENESIS OF ZEBRAFISH INNER EAR**

In vertebrates, all of the diverse cell types in the inner ear, as well as the neurons of statoacoustic ganglion, originate from a simple ectodermal thickening called the otic placode. In zebrafish, otic placode becomes morphologically visible adjacent to the hindbrain by 13.5 hpf (9 somite stage) (Fig. 1.3. A). Expression of various genes, including *pax8*, *pax2a* and *sox3*, mark preotic cells much earlier, starting around 9.5 hpf (Nikaido et al., 2007; Pfeffer et al., 1998; Phillips et al., 2001; Sun et al., 2007), which will be discussed in following sections. Later, the otic placode cavitates to become a fluid-filled hollow structure called the otic vesicle by 17-18 hpf (16-18 somite stage) (Fig. 1.3. B) (Haddon and Lewis, 1996). In birds and mammals, the otic vesicle forms by invagination rather than cavitation (Haddon and Lewis, 1996). Otoliths start to accumulate on the future utricular and saccular maculae (located at anterior and posterior end, Fig. 1.3 C) once the lumen of the otic vesicle forms. By 24 hpf, the first mature hair cells are visible in the utricular and saccular maculae, which continue to grow as the embryo grows into adulthood (Higgs et al., 2002; Riley et al., 1997). Semicircular canals and associated cristae start to form by 48 hpf, and keep developing as growth and morphogenesis of the otic vesicle continues (Fig. 1.3 D). The endolymphatic duct and lagenar macula develops much later by 8 - 10 days post-fertilization (Bever and Fekete, 2002; Riley and Moorman, 2000), and are not the focus of this study.



**Figure 1.3. Morphogenesis of the zebrafish inner ear. (Reprinted with permission from Kimmel et al., 1995)<sup>†</sup>**

(A) The otic placode (outlined by arrows) in a live embryo formed by 13.5 hpf. (B) At 18.5 hpf, the otic placode has cavitated to form the otic vesicle. The arrow pointing at the boundary between rhombomeres (segments of hindbrain) 4 and 5. (C) Otoliths (o) are prominent in the otic vesicle at 25 hpf. (D) A 60 hpf otic vesicle that has formed the primordia of the semicircular canals. Scale bar is 50  $\mu$ m for A-C, 100  $\mu$ m for D. Dorsal to the top, anterior to the left.

## DEVELOPMENTAL ORIGIN OF OTIC PLACODE, EPIBRANCHIAL PLACODE AND GANGLIA

The otic placode, along with various other cranial placodes, originates from a contiguous region called preplacodal ectoderm, a narrow band of cells wrapping around the anterior neural

<sup>†</sup> Figure 1.3 is reprinted with permission from "Stages of embryonic development of the zebrafish." by Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995, *Developmental Dynamics* 203, 253-310, Copyright 1995 by John Wiley & Sons.

plate. In all vertebrates, preplacodal ectoderm will segregate into a series of ectodermal patches called cranial placodes, including pituitary, nasal, lens, trigeminal, otic and epibranchial placodes (as well as lateral line placodes present in fish), surrounding anterior side of the developing central nervous system. Progenitor cells in these placodes will give rise to majority of neurons in various cranial ganglia and all the cells in special sense organs such as the olfactory epithelium, the lens and the inner ear (reviewed by Baker and Bronner-Fraser, 2001; Schlosser et al., 2014; Streit, 2004).

While the otic placode produces all of the cell types in the inner ear (as described in previous sections), developmentally related structures called epibranchial placodes are more developmentally constrained, producing a series of sensory neurons of the epibranchial ganglia. Epibranchial placodes form in close proximity to the otic placode and express many of the same genes (see later sections). Neuroblasts of epibranchial placodes begin to delaminate and migrate by 24 hpf, and by 36 hpf they differentiate into sensory neurons of the facial ganglion (cranial ganglion VII, also called geniculate), glossopharyngeal ganglion (cranial ganglion IX, or petrosal) and vagal ganglion (cranial ganglion X, or nodose). Epibranchial ganglia are positioned lateral to the otic vesicle, within the clefts between the pharyngeal arches (Baker and Bronner-Fraser, 2001). Neural crest derived precursors contribute Schwann cells and some later-forming sensory neurons to the proximal portions of epibranchial ganglia (Schlosser, 2006; Steventon et al., 2014). Neural crest cells are not required for induction of epibranchial placodes (Begbie et al., 1999), but are involved in helping the migration of placodal cells, formation and maintenance of epibranchial ganglia (Begbie and Graham, 2001; Culbertson et al., 2011; Steventon et al., 2014).

## INDUCTION OF OTIC AND EPIBRANCHIAL PLACODE

Although progenitors in otic and epibranchial placodes took on different fates and produce very different structures, they are induced by similar developmental signals, form in adjacent domains, and share some common early markers.

The otic placode has been much better-characterized in various species compared to epibranchial placodes. Many studies in different vertebrate model systems have shown that the otic placode is induced by members of the FGF family ligands secreted from adjacent hindbrain and subjacent mesoderm or endoderm, starting at the end of gastrulation until early segmentation stage (Alvarez et al., 2003; Freter et al., 2008; Ladher et al., 2005; Léger and Brand, 2002; Liu et al., 2003; Mansour et al., 1993; Maroon et al., 2002; Martin and Groves, 2006; Padanad et al., 2012; Park and Saint-Jeannet, 2008; Phillips et al., 2001; Wright and Mansour, 2003). In zebrafish, *Fgf3* and *Fgf8* are the primary otic inducers and act redundantly. Loss or knock-down of *fgf3* or *fgf8* alone leads to moderate deficiencies of otic tissue, while impairing functions of both *fgfs* results in much stronger deficiencies or complete loss of otic tissue (Léger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Phillips et al., 2001). Similar redundancy in *Fgfs* as otic inducers has since been demonstrated in other vertebrate species, although the primary inducing *Fgfs* are different. In mouse, *Fgf3* from the hindbrain and *Fgf10* from subjacent mesoderm act redundantly to induce otic placode (Alvarez et al., 2003; Wright and Mansour, 2003), while *Fgf8* appears to act indirectly as it regulates the expression of mesodermal *Fgf10* (Ladher et al., 2005). In chick, *Fgf3*, *Fgf8* and *Fgf19* are all involved in otic induction. Similar to mouse, *Fgf3* and *Fgf19* from the mesoderm are redundant otic inducers while *Fgf8* is required for proper mesodermal expression of *Fgf19* (Freter et al., 2008; Ladher et al., 2000; Ladher et al., 2005). Recent studies in zebrafish

showed a conserved role of Fgf10, as zebrafish ortholog Fgf10b expressed in the sub-otic mesoderm cooperates with Fgf3 and Fgf8 in inducing otic and epibranchial placodes (Maulding et al., 2014). Thus, induction of the otic placode is an evolutionary conserved process that requires cooperation of multiple Fgfs from multiple tissues.

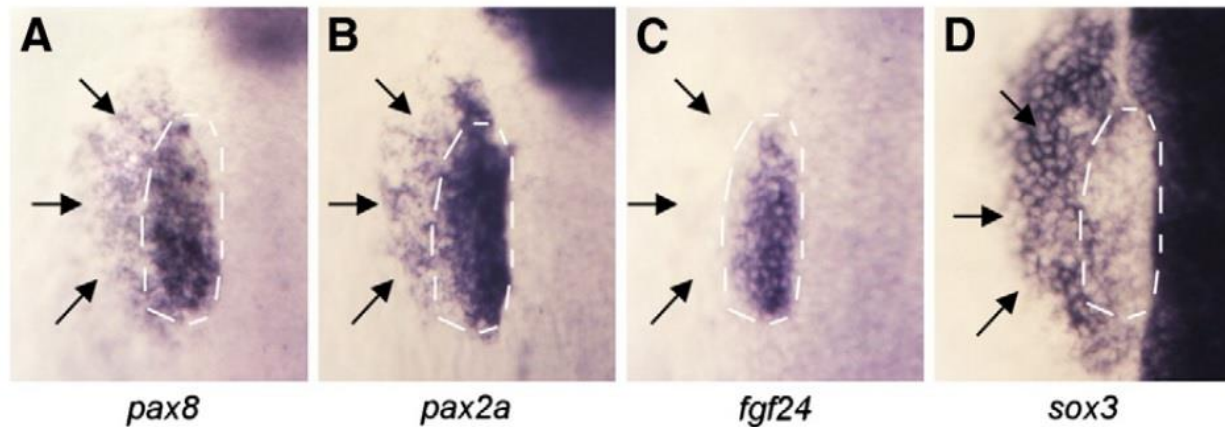
At slightly later stage, epibranchial placodes are also induced by Fgf3 and Fgf8 emanating from the hindbrain and subjacent mesoderm, developing lateral to otic placode (Nechiporuk et al., 2007; Nikaido et al., 2007; Sun et al., 2007). Blocking all Fgf signaling by pharmacological inhibitor SU5402 results in complete loss of otic and epibranchial placodes (Nikaido et al., 2007; Sun et al., 2007). Temporally controlled study revealed that induction of epibranchial placodes followed an anterior to posterior manner, requiring strong Fgf signaling between 10-16.5 hpf (Ladher et al., 2000). Similar to induction of otic placode, these Fgfs act in a redundant fashion. Epibranchial domains marked by *foxi1* and *pax2a* expression were absent or severely reduced in *fgf3;fgf8* double mutants, but were only slightly reduced in single mutants (Ladher et al., 2000). A recent study demonstrated that Fgf10b expressed in paraxial cephalic mesoderm is also involved in epibranchial development, as knocking down of *fgf10b* alone moderately impaired formation of epibranchial placodes and ganglia (Maulding et al., 2014). Moreover, disruption of *fgf10b* and *fgf3* together nearly abolished epibranchial domain and later eliminated epibranchial ganglia entirely (Maulding et al., 2014). In addition, the nascent otic placode is also a source of Fgf, emitting Fgf24 which is required for proper development of glossopharyngeal and vagal ganglia (Maulding et al., 2014; Padanad and Riley, 2011). After the inductive stage, Fgf3 and several Bmps expressed in the pharyngeal endoderm later signals the epibranchial placodes to initiate neurogenesis (Begbie et al., 1999; Holzschuh et al., 2005; Nechiporuk et al., 2005).

## MOLECULAR MARKERS OF OTIC PLACODE, EPIBRANCHIAL PLACODE AND GANGLIA

Although the otic placode only becomes morphologically visible by 13.5 hpf (9 somite stage), several transcription factors, including *pax8*, *pax2* and *sox3*, are expressed early in the pre-otic region by 9 hpf (late gastrula stage) in response to Fgf signals (Nikaido et al., 2007; Pfeffer et al., 1998; Phillips et al., 2001; Sun et al., 2007). Expression of *pax8* and *sox3* are the earliest markers for otic development (Nikaido et al., 2007; Pfeffer et al., 1998; Phillips et al., 2001; Sun et al., 2007). Later, *pax2a*, a closely related homolog of *pax8* and one of the two *pax2* paralogs in zebrafish, starts to express in the otic anlagen by 11 hpf (3 somite stage) (Pfeffer et al., 1998). Another *pax2* paralog, *pax2b*, appears in the otic cells later around 13.5 hpf (9 somite stage), just before the morphological thickening of otic placode, and follows the expression pattern of *pax2a* later but at lower expression levels (Pfeffer et al., 1998). Expression of *pax8* is gradually downregulated as the placode forms and eventually disappears from the nascent otic vesicle by 19 hpf (20 somite stage) (Pfeffer et al., 1998). Expression of *pax2a*, on the other hand, persists throughout otic development, albeit localized to sensory epithelia and medial side of the otic vesicle at later stages (Riley et al., 1999). *sox3* can also be detected throughout otic development (Nikaido et al., 2007) (later sections of Chapter I and Chapter III), although its expression pattern becomes highly dynamic starting at 12 hpf, when it is downregulated to a lower level in the pre-otic region and upregulated in the nascent epibranchial domain (Fig. 1.4 D) (Nikaido et al., 2007; Padanad and Riley, 2011). Expression of *sox3* will be discussed in detail in later sections of this chapter and Chapters II and III.

Early detection of the epibranchial domain relies on the same molecular markers, *pax8*, *sox3* and *pax2a*, whereas morphological thickening of epibranchial placodes is only detectable by 24 hpf (Maulding et al., 2014; Nechiporuk et al., 2007; Nechiporuk et al., 2005; Nikaido et al., 2007; Padanad and Riley, 2011; Sun et al., 2007). Soon after otic induction, epibranchial progenitors form in an arc-shaped region wrapping laterally around the nascent preotic domain, marked by high-level expression of *sox3* and low-level expression of *pax8* and *pax2a* in comparison to the otic region (Fig. 1.4 A, C, D) (Padanad and Riley, 2011). Expression of *pax8* is later lost in the epibranchial domain. By 14 hpf (10 somite stage) epibranchial expression of *sox3* starts to elongate along the anterior-posterior axis, and later segregates into three subdomains by 19 hpf (20 somite stage) (Nikaido et al., 2007). They are located lateral to the otic vesicle, representing the nascent facial, glossopharyngeal and vagal placode (Nikaido et al., 2007). *pax2a* is also expressed in individual epibranchial placodes starting from 16-18 hpf (Maulding et al., 2014; Nechiporuk et al., 2007). After the onset of neurogenesis at 24 hpf, proneural genes *neurog1* and *neuroD* are expressed in neuroblasts of epibranchial placodes (Andermann et al., 2002). As epibranchial neuronal precursors differentiate, they express homeobox transcription factors *phox2a* and *phox2b*, and later condense together with neuro-crest-derived Schwann cells to form epibranchial ganglia (Begbie et al., 1999; Guo et al., 1999; Nechiporuk et al., 2007).





**Figure 1.4. Otic and epibranchial markers.** (Reprinted with permission from Padanad and Riley, 2011)<sup>‡</sup>

Dorsal views of expression of *pax8* (A), *pax2a* (B), *fgf24* (C) and *sox3* (D) at 12 hpf in wild-type embryos. Otic regions are outlined by white dashed lines, while epibranchial regions are indicated by black arrows.

## SOX PROTEIN FAMILY

Members of the SOX family proteins exist widely throughout the animal kingdom (Bowles et al., 2000; Soullier et al., 1999; Wegner, 1999). They are transcription factors characterized by a HMG (high-mobility-group) DNA-binding domain that is conventionally at least 50% identical to the HMG domain of SRY, the mammalian male-determining factor and founding member of the SOX family (Bowles et al., 2000; Gubbay et al., 1990; Sinclair et al., 1990). Therefore, they are named as Sry-related HMG-box (SOX) proteins. All HMG domain containing proteins are in a superfamily, which divides into two subfamilies, TCF/SOX/MATA group, typically containing only one sequence-specific HMG box, and HMG/UBF group, bearing multiple HMG boxes that

<sup>‡</sup> Figure 1.4 is reprinted with permission from “Pax2/8 proteins coordinate sequential induction of otic and epibranchial placodes through differential regulation of *foxi1*, *sox3* and *fgf24*.” by Padanad, M.S., Riley, B.B., 2011, *Developmental Biology* 351, 90-98, Copyright 2011 by Elsevier.

are less sequence-specific (Grosschedl et al., 1994; Laudet et al., 1993; Soullier et al., 1999). SRY and SOX are in the former group. Based on sequence comparison in HMG domain as well as full-length protein structure, SOX family proteins are further categorized into groups A-J (Bowles et al., 2000). Members of the same group usually share high degree of amino acid identity in their HMG domain (Wegner, 1999).

## **SOXB1 TRANSCRIPTION FACTORS IN THE INNER EAR**

Sox2 and Sox3 are members of the group B1 Sox transcription factor family (SoxB1 family) that are conserved among vertebrates. The zebrafish SoxB1 family comprises Sox1a/b, Sox2, Sox3 and Sox19a/b, whereas the amniotes SoxB1 family includes Sox1, Sox2 and Sox3 (Okuda et al., 2006). A previous study comparing the expression patterns of these four SoxB1 genes in zebrafish showed that only *sox2* and *sox3* are expressed in the otic region (Okuda et al., 2006). Indeed, *sox2* and *sox3* are among the earliest markers of otic development (see previous sections in this chapter and Chapter II). The expression of *sox2* starts around 12.5 hpf in the otic region (this study, Chapter II), and by 13-14 hpf forms two domains along the medial edge of the pre-placode at the anterior and posterior end, marking the future utricular and saccular maculae respectively (Millimaki et al., 2010). At otic vesicle stage, expression of *sox2* marks the developing sensory epithelium. As sensory epithelia mature, *sox2* expression is lost from hair cells but persists in support cells (Millimaki et al., 2010). Expression of *sox2* is regulated by Fgf, Atoh1a/b and Notch (Millimaki et al., 2010).

*sox3* is one of the earliest otic/epibranchial markers, induced by nearby Fgf signals and the local pre-placodal ectoderm marker *foxi1* (Nikaido et al., 2007; Padanad and Riley, 2011; Sun et

al., 2007). Its expression pattern is highly dynamic and sensitive to Fgf levels. Rising Fgf levels caused by Fgf24 emanating from the nascent otic placode downregulate *sox3* in the otic region to a discrete lower level, while it upregulates *sox3* in the nascent epibranchial domain (Padanad and Riley, 2011). Otic expression of *sox3* persists at a lower level throughout the placode and in the floor of the otic vesicle, overlapping with *sox2* in the sensory domain (medial half of the floor) and the proneural factor *neurog1* (in the lateral half of the floor) (this study, Chapter III). Expression and function of *sox3* in the inner ear, and its relationship to *sox2*, is described in Chapter III.

SoxB1 transcription factors are implicated in various developmental events, especially neural development. They are early markers of the neural ectoderm during gastrulation and are crucial for the development of the central nervous system (Ferri et al., 2004; Graham et al., 2003; Kishi et al., 2000; Penzel et al., 1997; Pevny et al., 1998). Sox2 is well known to regulate stem cell pluripotency and was one of the four factors shown to induce pluripotent stem cells from differentiated cells (Takahashi and Yamanaka, 2006). Sox2 is also important for proper development of sensory organs, for example the lens (Kamachi et al., 2001) and the inner ear (Kiernan et al., 2005; Millimaki et al., 2010). Less attention was focused on Sox3, as often it is thought to be redundant in tissues where it is co-expressed with other SoxB1 factors (Collignon et al., 1996; Graham et al., 2003; Overton et al., 2002; Wood and Episkopou, 1999). However, functional diversification of SoxB1 transcription factors can be achieved through regulation of SoxB1 expression levels and interaction with co-factors. Both these variables will be examined in Chapters II and III.

Sox2 and Sox3 are both expressed in the developing inner ear in partially overlapping domains, from early placodal stage to at least 36 hpf otic vesicle stage (latest examined), indicating they might have different roles in the inner ear during early and late developmental processes.

Sox2 is essential for neurosensory development in mouse and chick inner ear (Evsen et al., 2011; Kiernan et al., 2005). Previous work in our lab also showed that *sox2* is required for maintenance and regeneration of hair cells in the zebrafish inner ear (Millimaki et al., 2010). Sox3 has been implicated in neural development of the chick inner ear (Abello et al., 2010). However, Sox2/3's full range of functions, factors that influence their function, as well as to what degree their functions overlap remains obscure during inner ear development. These will be addressed in Chapters II and III in this study.

## CHAPTER II

### SOX2 AND SOX3 COOPERATE TO REGULATE OTIC/EPIBRANCHIAL PLACODE INDUCTION IN ZEBRAFISH<sup>§</sup>

#### INTRODUCTION

The otic placode, the precursor of the inner ear, is induced by Fgfs emanating from the hindbrain and subjacent mesendoderm during a period lasting from late gastrulation through early segmentation (Alvarez et al., 2003; Freter et al., 2008; Ladher et al., 2005; Léger and Brand, 2002; Liu et al., 2003; Mansour et al., 1993; Maroon et al., 2002; Martin and Groves, 2006; Maulding et al., 2014; Padanad et al., 2012; Park and Saint-Jeannet, 2008; Wright and Mansour, 2003). After a brief lag, epibranchial placodes are also induced by Fgf and share many of the same early markers. In zebrafish, *pax8* and *sox3* are the earliest markers of otic and epibranchial development (Nikaido et al., 2007; Phillips et al., 2001; Sun et al., 2007). Loss-of-function and overexpression studies show that *pax8* helps initiate otic differentiation and, together with *pax2a* and *pax2b*, maintains otic fate as the placode gives rise to the otic vesicle (Hans et al., 2004; Ikenaga et al., 2011; Mackereth et al., 2005; Padanad et al., 2012). Loss of *pax2/8* function does not block epibranchial placode formation (Mackereth et al., 2005) but does impair subsequent differentiation of epibranchial ganglia (Padanad and Riley, 2011). The role of *sox3* in otic/epibranchial development is less well defined and has heretofore been examined primarily using morpholino

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<sup>§</sup> Reprinted with permission from article: Gou, Y., Guo, J., Maulding, K., Riley, B.B., 2018. *sox2* and *sox3* cooperate to regulate otic/epibranchial placode induction in zebrafish. *Developmental Biology* 435, 84-95. Copyright 2018 by Elsevier.

oligomer (MO)-mediated gene knockdown (Padanad and Riley, 2011). Knockdown of either *sox3* or *pax8* causes moderate reduction in the size of the otic placode, whereas knockdown of both *sox3* and *pax8* causes a more severe reduction. Similarly, knockdown of *sox3* causes partial loss of epibranchial ganglia and simultaneous knockdown of *pax8* strongly enhances this phenotype. These data suggest that *pax8* and *sox3* each provide some non-overlapping functions required for early otic differentiation. However, concerns over possible off-target effects of MOs highlight the need to analyze gene knockouts in order to corroborate or refine previous findings (Kok et al., 2015; Rossi et al., 2015; Schulte-Merker and Stainier, 2014).

Expression of *sox3* is regulated in a dynamic fashion by changing levels of Fgf. Initially *sox3* is induced at a relatively high level in otic cells in the early response to Fgf (Nikaido et al., 2007; Sun et al., 2007). Subsequently, *sox3* expression declines to a discrete lower level in the otic placode in response to locally rising Fgf levels (Bhat and Riley, 2011; Maulding et al., 2014; Padanad and Riley, 2011). At the same time *sox3* is induced at a relatively high level in prospective epibranchial cells abutting the lateral edge of the otic placode. The significance of reducing *sox3* expression in the otic placode is not known but potentially reflects a requirement for developmental progression. Sox3 is a member of the SoxB1 family of transcription factors, which are known to maintain pluripotency of progenitors or promote early stages of differentiation, depending on the level of expression (Gómez-López et al., 2011; Hutton and Pevny, 2011; Juuri et al., 2012; Kondoh and Kamachi, 2010; Matsushima et al., 2011; Packard et al., 2016; Rizzino and Wuebben, 2016; Tucker et al., 2010). Accordingly, dynamic regulation of *sox3* might modulate the balance of pluripotency vs. differentiation during early otic development.

Another variable that could influence *sox3* function is overlapping expression of the related gene *sox2*. We previously reported that otic expression of *sox2* begins at 14 hpf (10 somite stage) in association with early development of sensory epithelia (Millimaki et al., 2010). However, we recently discovered that low-level expression of *sox2* can be detected in the otic/epibranchial region as early as 12–12.5 hpf (6 somite stage). This raises the possibility that *sox2* provides some degree of redundancy with *sox3* during otic/epibranchial placode induction, and it could also affect the balance of pluripotency vs. differentiation by elevating overall SoxB1 levels.

To address the above issues, we generated knockout lines and heat shock-inducible transgenic lines for *sox2* and *sox3* to evaluate their functions in otic and epibranchial placode development. Knockouts of *sox2* and *sox3* cause phenotypes that closely mimic their respective MO phenotypes. Mutant analysis confirms that *sox2* and *sox3* provide partially redundant functions required for establishing a normal amount of otic and epibranchial tissue. Weak misexpression of *sox2* can rescue placodal deficiencies caused by loss of *sox3*, consistent with functional redundancy. However, strong misexpression of either *sox2* or *sox3* cell-autonomously blocks initial stages of otic differentiation, confirming that the function of these genes is concentration-dependent. We also confirm that *pax8*<sup>-/-</sup> mutants (like *pax8*-morphants) show a more severe placodal deficiency than *sox3*<sup>-/-</sup> mutants, but the placodal deficiency is no worse in *pax8*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants. This suggests that *pax8* and *sox3* work together in the same pathway rather than providing distinct gene-specific functions.

## MATERIALS AND METHODS

### Fish strains and developmental conditions

The wild-type strain was derived from the AB line (Eugene, OR). The *pax2a* mutant allele *noi<sup>tu29a</sup>* (Brand et al., 1996; Lun and Brand, 1998) and *pax8* mutant allele *pax8-RFP* (Ikenaga et al., 2011) are referred to herein as *pax2a*<sup>-/-</sup> and *pax8*<sup>-/-</sup>, respectively. Mutant alleles *sox2<sup>x50</sup>*, *sox3<sup>x52</sup>* and *sox3<sup>x53</sup>* were generated by TALEN and CRISPR/Cas9 technology. TALEN left and right arm constructs were assembled using GoldyTALEN (Bedell et al., 2012), sequences of which are highlighted blue in Fig. 2.1A. For targeting *sox3*, single strand guide RNA (blue sequence in Fig. 2.1A) and Cas9 mRNA were co-injected into one-cell stage wild-type embryos that were raised for screening founders. Transgenic lines *TG(hsp70:fgf8a)<sup>x17</sup>*, *TG(hsp70:sox2)<sup>x21</sup>* (Millimaki et al., 2010) and *TG(hsp70:sox3)<sup>x32</sup>* (first described here) were used for gene misexpression and referred to as *hs:fgf8*, *hs:sox2* and *hs:sox3*, respectively. *TG(brn3c: gap43-GFP)* (Xiao et al., 2005) was used to visualize sensory hair cells. Embryos were developed under standard conditions at 28.5 °C (except for heat-shock experiments as noted below) in fish water containing methylene blue and staged based on standard morphological features (Kimmel et al., 1995). Young embryos were co-stained for *myoD* during in situ hybridization to count somites for precise staging. PTU (1-phenyl 2-thiourea, 0.3 mg/ml) was supplemented to fish water to prevent melanin formation in older embryos (>24 hpf).

### Gene misexpression and morpholino injections

Misexpression was accomplished by briefly incubating embryos heterozygous or homozygous for heat-shock inducible transgenes in a water bath at 39 °C for 30 or 60 min (except



where noted in the text). Embryos were developed at 33 °C after heat-shock until fixation. Wild-type embryos were also heat shocked to serve as controls in all misexpression studies. To knock down *sox3*, wild-type embryos were injected at the one-cell stage with varying doses of *sox3*-mol (Okuda et al., 2010), as indicated in the text. In all morpholino knock-down experiments, *p53*-mo (Robu et al., 2007) was co-injected to prevent non-specific cell death. Phenotypes described in this study were examined in at least 15 embryos per probe and time point unless stated otherwise.

### **In situ hybridization, cell transplantation and data analysis**

Whole-mount in situ hybridization was performed as previously described (Phillips et al., 2001). Transgenic donor embryos were injected with lineage tracer (10,000 MW, lysine-fixable tetramethylrhodamine labeled dextran in 0.2 M KCl) at the one-cell stage. Donor cells were then transplanted into non-labeled wild-type host embryos at the late blastula-stage. Quantification of otic vesicle size and gene expression area was performed using Photoshop measuring the number of pixels, and data were normalized relative to wild-type control embryos. The edges of gene expression domains were determined by comparing stained embryos viewed at high magnification with corresponding images in Photoshop. Quantification of anterior expansion distance of ectopic *pax8*-expressing cells was performed using Photoshop by measuring distances in pixels, then converting to microns based on the fold-magnification of the images. Significance was evaluated by students' t-test (pair-wise comparison), or ANOVA and Tukey's post-hoc HSD tests (for experiments involving more than two groups).

## RNA extraction, cDNA synthesis and qRT-PCR

For each genotype, RNA was extracted from 24 embryos at 36 hpf using Trizol (Life Technologies) and chloroform (MACRON fine chemicals). cDNA were synthesized from 1.5 µg of total RNA using SuperScript First-strand synthesis kit (Invitrogen). To generate a linear range of PCR products, cDNA samples were diluted 1:64 for *sox2* and *sox3* specific amplification, and 1:2048 for *b-actin* (a constitutive control). For each independent experiment, template dilutions were done in triplicate, and each experiment was repeated three times. 10 µl reaction mixtures including PowerUp SYBR green master mix (Applied Biosystems), diluted cDNA and gene specific primers in the ratio of 5:3:2 were dispensed into 96-well plates with optical adhesive covers (Bio-Rad), and run in an Applied Biosystems 7300 real time PCR system using the default protocol. Fold-change for each gene was calculated using the  $2^{-\Delta\Delta C_T}$  method. Results from three independent experiments were averaged to calculate means and standard error of means. Data were presented as fold-changes relative to wild-type control mRNA levels. Gene specific primer pairs used for qRT-PCR are as follows: *sox2* 5'-AACGGCTCGCCACCTA-3' and 5'-TCATTCCCGGCGTGCTT-3'; *sox3* 5'-GCCGACCACTCCAGTCTACA-3' and 5'-CTGTCCCTGCGCTTTGATAGT-3'; *b-actin* 5'-AGGTCATCACCATCGGCAAT-3' and 5'-CAATGAAGGAAGGCTGGAACAG-3'. *sox2*<sup>-/-</sup> homozygous mutant embryos were identified by their characteristic tail phenotype (Fig. 2.1L) and a deficiency in hair cell accumulation (Gou et al., 2018).

## PCR genotyping

To ensure accurate identification of *sox2*<sup>-/-</sup>, *sox3*<sup>-/-</sup> and *pax8*<sup>-/-</sup> mutants, individual embryos were prepared for single-embryo genotyping after phenotype or expression patterns were

documented. DNA from single embryos was extracted following methods described previously (Meeker et al., 2007), with addition of a proteinase-K digestion step for embryos older than 12 hpf or fixed embryos processed by in situ hybridization. *sox2* homozygous and heterozygous mutants were identified by PCR using forward primer 5'-CCAGCAAAGTTACCTCCAAGT-3' and reverse primer 5'-GCAGGGTGTACTTGTCTTCTT-3'. PCR products were then digested with restriction enzyme SfoI or NarI (NEB), yielding wild-type fragments of 330 and 160 bp, while the mutant PCR product remains uncut at 490 bp. To identify *sox3<sup>x52</sup>* mutants, indel-PCR was performed using three primers in a single reaction: forward primer-1 5'-CGTTTTCTTTCGAGTGCTTGGC-3', forward primer-2 (indel primer) 5'-GCAAAAACAACAGTGCCAACGA-3' and reverse primer 5'-TTTGTAATCCGGGTGCTCCTTC-3'. *sox3<sup>x52/x52</sup>* homozygous mutant DNA yielded a single 344 bp amplicon, while wild-type or *sox3<sup>x52/+</sup>* heterozygous DNA yielded 239 and 344 bp fragments. To identify *pax8-RFP* mutants, forward primer 5'-TCTTCACCCTCACCAGAAATGACC-3' and reverse primer 5'-ATTGTGTGCATTTATCAGCGCAGTG-3' flanking the DsRed Express insert were used. PCR yielded a ~1.2 kb fragment in *pax8-RFP* homozygous mutants, and a ~300 bp fragment in wild-type embryos.

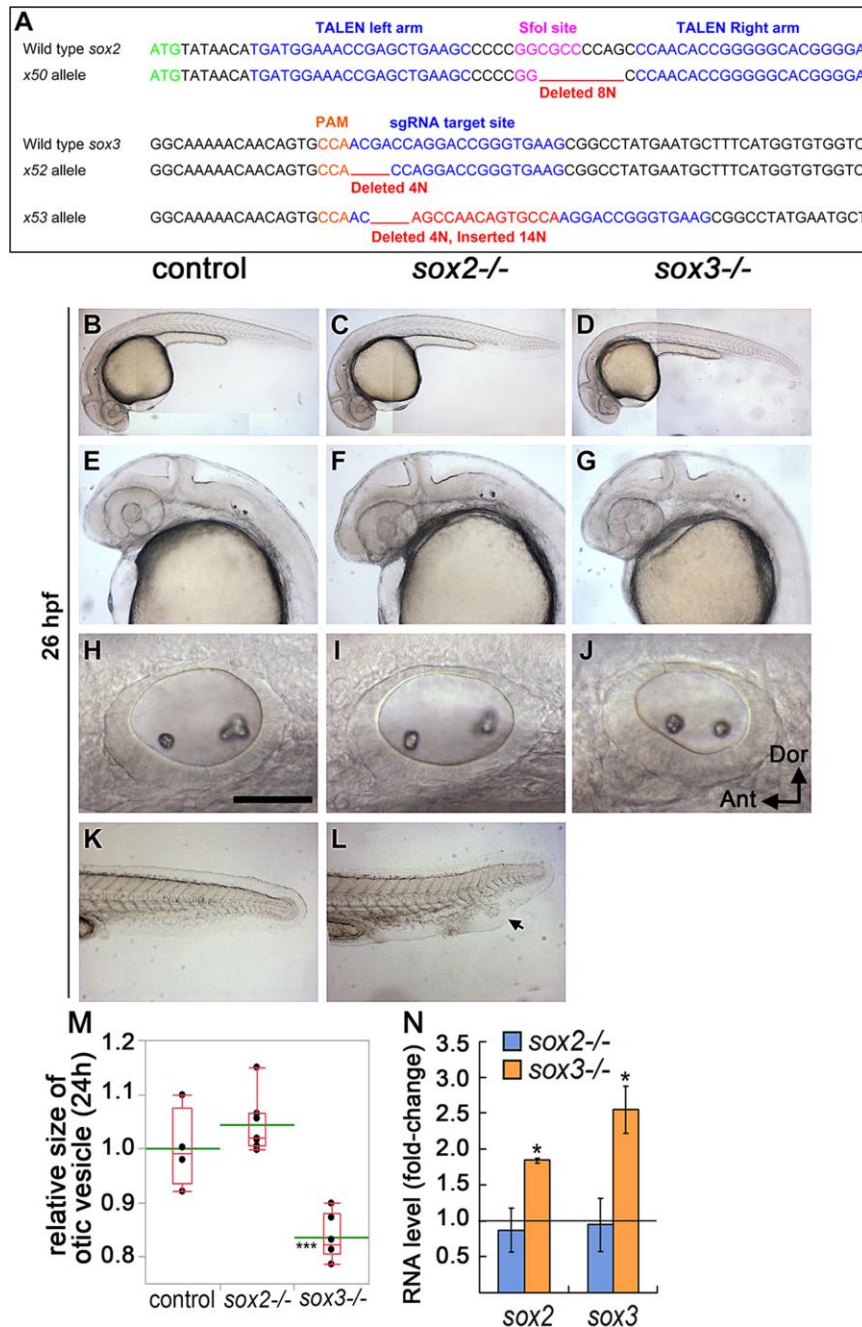
## RESULTS

### Targeting *sox2* and *sox3*

To evaluate the functions of *sox2* and *sox3* in otic/epibranchial induction, we used TALEN and CRISPR/Cas9 technology to target *sox2* and *sox3* (Fig. 2.1A and Materials and Methods). We

recovered one lesion for *sox2* (allele *x50*) and two lesions for *sox3* (alleles *x52* and *x53*) that lead to frame shifts near the 5' end of the gene followed by multiple premature stop codons (presumptive null mutations, Fig. 2.1A). Phenotypes of *sox3*<sup>*x52/x52*</sup> and *sox3*<sup>*x53/x53*</sup> are identical, but for simplicity all figures in this paper depict data obtained with *sox3*<sup>*x52/x52*</sup> mutants, hereafter referred to as *sox3*<sup>-/-</sup> mutants. Both *sox2*<sup>-/-</sup> and *sox3*<sup>-/-</sup> mutants show largely normal morphology at 26 hpf (Fig. 2.1B-G) except that *sox2*<sup>-/-</sup> mutants show minor growth defects at ventral tip of the tail (Fig. 2.1L arrow) and *sox3*<sup>-/-</sup> mutants show a 15–20% reduction in the size of the otic vesicle (Fig. 2.1J, M). During subsequent development *sox2*<sup>-/-</sup> mutants fail to inflate their swim bladders and die around 7–9 dpf, whereas *sox3*<sup>-/-</sup> mutants subsequently recover and grow into healthy fertile adults with no obvious morphological or behavioral abnormalities.

Because such mutations sometimes lead to nonsense-mediated decay of mutant transcripts, we performed quantitation of *sox2* and *sox3* transcript levels in mutant embryos. *sox2*<sup>-/-</sup> mutants show no significant changes in accumulation of *sox2* or *sox3* transcript levels (Fig. 2.1N). Surprisingly, *sox3*<sup>-/-</sup> mutants show a nearly two-fold increase in *sox2* transcript levels and a 2.5-fold increase in *sox3* transcript levels (Fig. 2.1N). Elevation of *sox2* mRNA levels in *sox3*<sup>-/-</sup> mutant could ameliorate the phenotype, possibly contributing to their ability to recover and survive.



**Figure 2.1. Genetic targeting of *sox2* and *sox3*.**

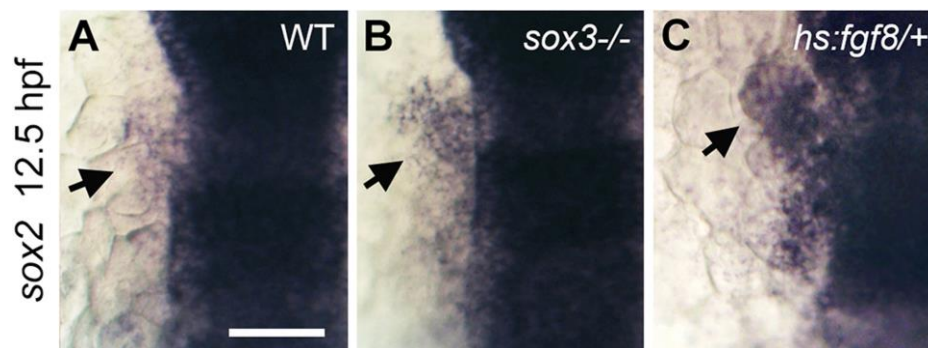
(A) Sequences for targeting vectors for *sox2* (TALEN) and *sox3* (sgRNA) and resulting lesions in *sox2* (allele *x50*) and *sox3* (alleles *x52* and *x53*). The deletion in *x50* leads to loss of a SfoI restriction site (magenta), which was used for genotyping, whereas *sox3* indels were identified by allele-specific PCR primers (see Materials & Methods). (B-L) Live embryos at 26 hpf showing general morphology (B-D), cranial development (E-G), tail development (K-L) and the otic vesicle (H-J, lateral views with anterior to the left; Scale bar, 50  $\mu$ m) in control, *sox2*<sup>-/-</sup> and *sox3*<sup>-/-</sup> mutants. Excess cells at the ventral tip of the tail in *sox2*<sup>-/-</sup> embryos are indicated (arrow).

### Figure 2.1. Continued.

(M) Box-and-whisker plot of surface area of otic vesicle, normalized to control embryos, in control embryos and *sox2*<sup>-/-</sup> and *sox3*<sup>-/-</sup> mutants. Green line indicates mean. Asterisk indicates statistically significant difference relative to control embryos (\*\*\*  $P < 0.001$ , Tukey's HSD test). (N) Quantitative real time PCR measurements of fold changes in *sox2* and *sox3* mRNA levels in *sox2*<sup>-/-</sup> and *sox3*<sup>-/-</sup> mutants at 36 hpf normalized to wild-type control embryos. Error bars represent standard error of the means. Asterisks indicate statistically significant differences relative to controls (\*  $P < 0.05$ , t-tests).

### Early placodal expression of *sox2*

We previously reported that otic expression of *sox2* begins around 14 hpf (10 somite-stage), just after the otic placode becomes morphologically visible (Millimaki et al., 2010). However, subsequent analysis revealed that weak otic/epibranchial expression of *sox2* can be detected in up to a third of wild-type embryos by 12–12.5 hpf (6–7 somites stage) (Fig. 2.2A). In *sox3*<sup>-/-</sup> mutants the onset of otic/epibranchial expression of *sox2* was normal, but expression was more intense and fully penetrant (Fig. 2.2B), consistent with higher *sox2* transcript abundance in this background. Similarly, the intensity of early expression of *sox2* increased in all embryos following misexpression of *fgf8* at 10 hpf (Fig. 2.2C). These findings are consistent with the possibility that *sox2* cooperates with *sox3* in regulating early otic/epibranchial development.



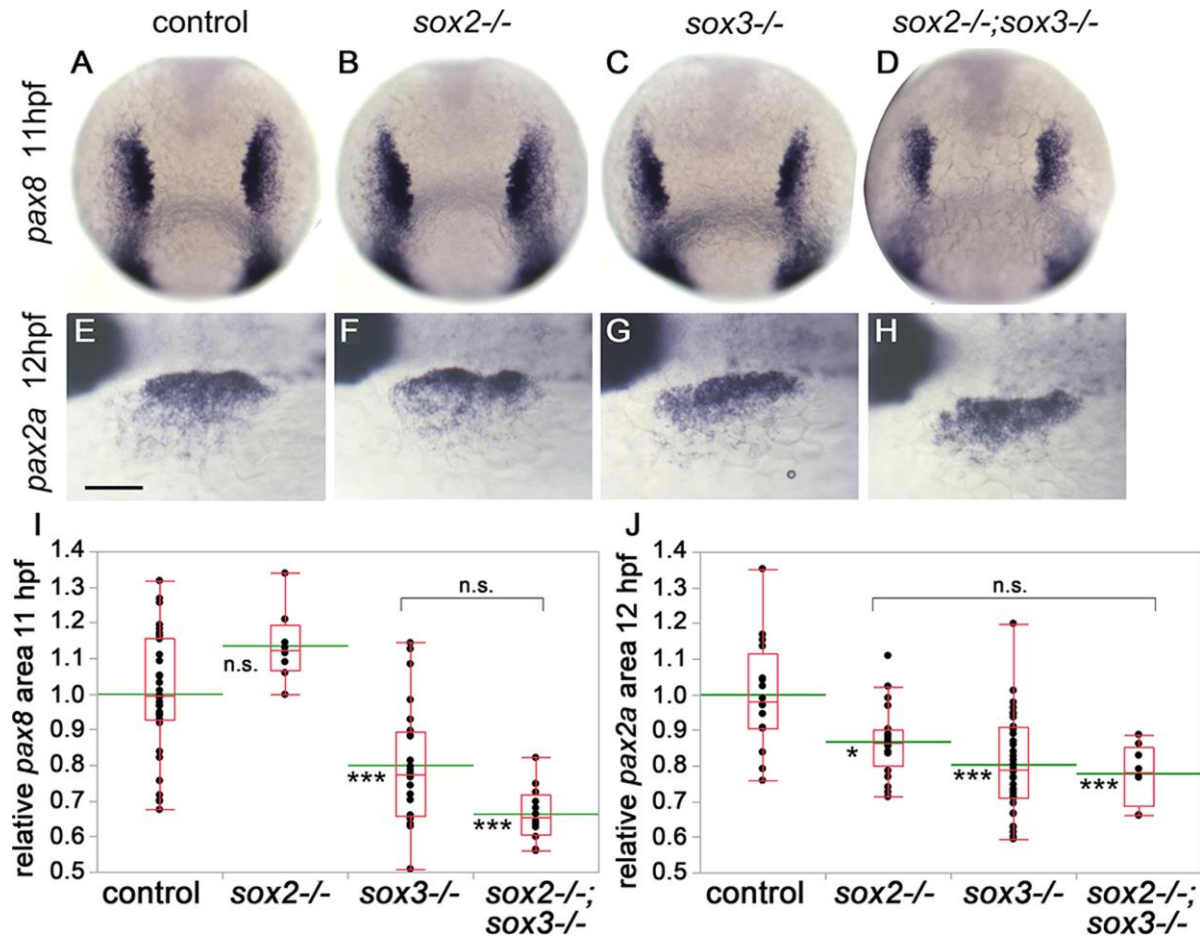
**Figure 2.2. Early placodal expression of *sox2*.**

**Figure 2.2. Continued.**

Otic/epibranchial *sox2* expression (arrows) at 12.5 hpf in a wild-type embryo (A), a *sox3*<sup>-/-</sup> mutant (B) and a *hs:fgf8*/<sup>+</sup> transgenic embryo (C) heat shocked at 10 hpf, 39 °C for 30 min. Dorsal views with anterior up, lateral to the left. Scale bar, 100 μm.

**Effect of loss of *sox2* and *sox3* in early otic and epibranchial placode**

In close agreement with the effects of morpholino knockdown of *sox3* (Padanad and Riley, 2011), *sox3*<sup>-/-</sup> mutants show a 20% decrease in the otic domain of *pax8* at 11 hpf and a similar decrease in the otic domain of *pax2a* at 12 hpf (Fig. 2.3C, G, I, J). The deficiency in otic tissue persists through at least 26 hpf (Fig. 2.1J), confirming that *sox3* is required for normal otic induction. In *sox2*<sup>-/-</sup> mutants, otic expression of *pax8* is normal whereas the domain of *pax2a* at 12 hpf is reduced by about 15% (Fig. 2.3B, F, I, J), consistent with the later onset of *sox2* expression. This shows that *sox2* is also required for normal otic induction, although *sox2*<sup>-/-</sup> mutants recover to form a morphologically normal otic vesicle by 26 hpf (Fig. 2.1I). In *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants, the otic domain of *pax8* is reduced by 33% (Fig. 2.3D, I), although this deficiency is not significantly worse than in *sox3*<sup>-/-</sup> mutants (p=0.06). Likewise, the otic domain of *pax2a* is reduced by 22% in *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants, which is similar to the deficiency in *sox3*<sup>-/-</sup> mutants (Fig. 2.3H, J). Thus, the weak early expression of *sox2* has little effect on early otic development in the absence of *sox3*. Additionally, the two-fold increase in *sox2* transcript accumulation seen in *sox3*<sup>-/-</sup> mutants apparently does not ameliorate the early deficiency in otic development.



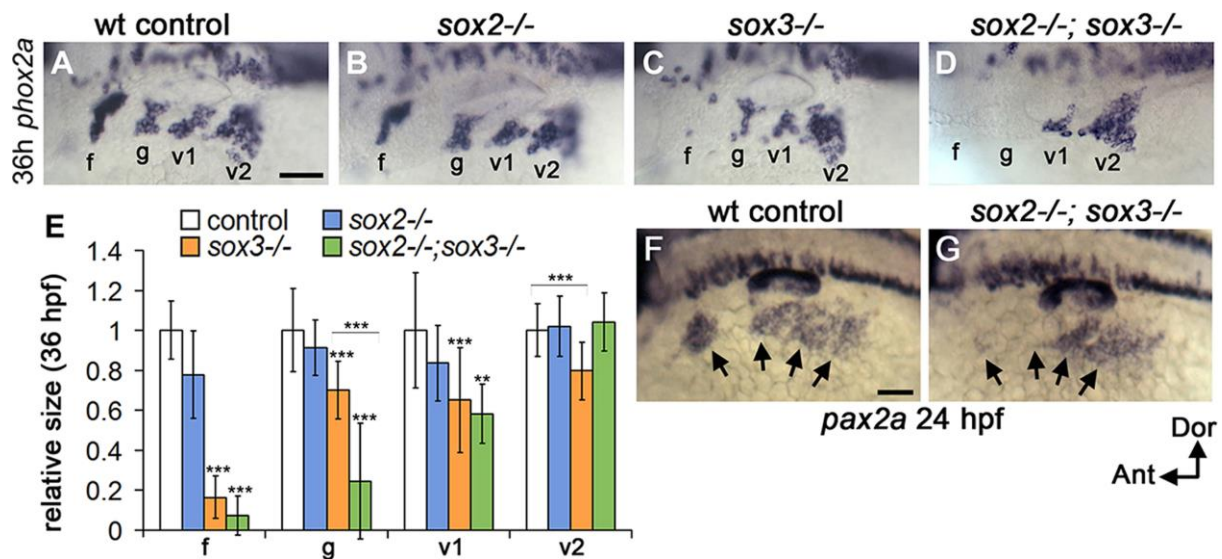
**Figure 2.3. Effect of *sox2*<sup>-/-</sup> and *sox3*<sup>-/-</sup> on early placode size.**

(A-H) Expression of *pax8* at 11 hpf (A-D) and *pax2a* at 12 hpf (E-H) in control embryos (A, E), *sox2*<sup>-/-</sup> mutants (B, F), *sox3*<sup>-/-</sup> mutants (C, G) and *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants (D, H). Dorsal views with anterior up (A-D) or dorsal-lateral views with anterior to the left (E-H). (I, J) Box-and-whisker plots of relative surface area of otic/epibranchial domain of *pax8* (I) or *pax2a* (J) in control, *sox2*<sup>-/-</sup>, *sox3*<sup>-/-</sup> and *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutant embryos. Data are normalized relative to control groups, with means indicated by green lines. Asterisks indicate statistically significant differences relative to control (\**P*<0.05, \*\*\* *P*<0.001, Tukey's HSD test). Brackets indicate comparisons between non-control groups. n.s., not significantly different.

We next examined development of epibranchial ganglia at 36 hpf, marked by expression of *phox2a* (Begbie et al., 1999; Lee et al., 2003; Nechiporuk et al., 2005). In keeping with previous *sox3* knockdown experiments (Padanad and Riley, 2011), *sox3*<sup>-/-</sup> mutants show deficiencies in all epibranchial ganglia at 36 hpf, especially in the facial ganglion which was reduced by 80% (Fig.



2.4C, E). *sox2*<sup>-/-</sup> mutants showed slight but non-significant deficiencies in epibranchial ganglia (Fig. 2.4B, E). However, *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants show much more pronounced deficiencies compared to *sox3*<sup>-/-</sup> mutants (Fig. 2.4D, E). For example, some *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants showed complete ablation of the facial (3/6 specimens) and glossopharyngeal ganglia (1/6 specimens). We also examined expression of *pax2a* at 24 hpf, which marks epibranchial placodes prior to the onset of neurogenesis. While wild-type embryos show four discrete patches of *pax2a* expression, *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants show marked deficiencies in anterior patches of *pax2a* expression corresponding to facial and glossopharyngeal placodes (Fig. 2.4F, G). Together, these data show that *sox2* and *sox3* cooperate and are required to establish a full complement of both otic and epibranchial placodal tissue.



**Figure 2.4. Effect of *sox2*<sup>-/-</sup> and *sox3*<sup>-/-</sup> on epibranchial ganglia.**

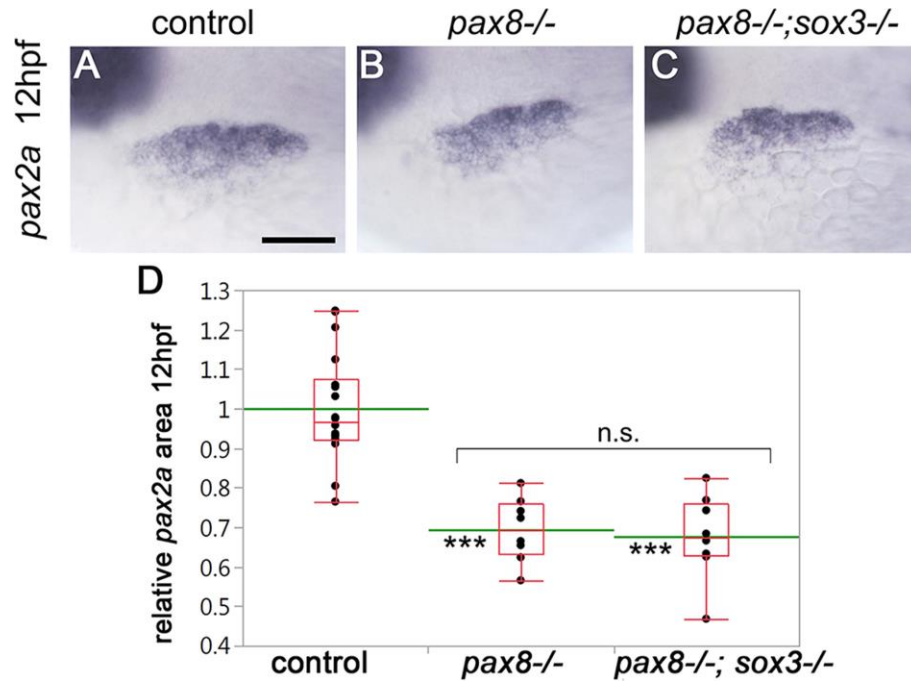
(A-D) Expression of *phox2a* at 36 hpf in a wild-type embryo (A), *sox2* (B), *sox3*<sup>-/-</sup> (C) and *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutant (D) embryo. Locations of the facial (f), glossopharyngeal (g), and vagal (v1 and v2) ganglia are indicated. Dorsal-lateral views with anterior to the left. (E) Quantitation of relative surface area of *phox2a* expression in individual epibranchial ganglia in control, *sox2*<sup>-/-</sup>, *sox3*<sup>-/-</sup> and *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutant embryos. Data show means and standard deviations normalized to wild-type controls. Asterisks indicate statistically significant differences relative to controls (\*\* *P*<0.01, \*\*\* *P*<0.001, Tukey's HSD test), or between non-

**Figure 2.4. Continued.**

control groups (bracket). (F, G) Dorsolateral views (anterior to left) showing expression of *pax2a* at 24 hpf in a control embryo (F) and *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutant (G). Arrows indicate positions of epibranchial placodes.

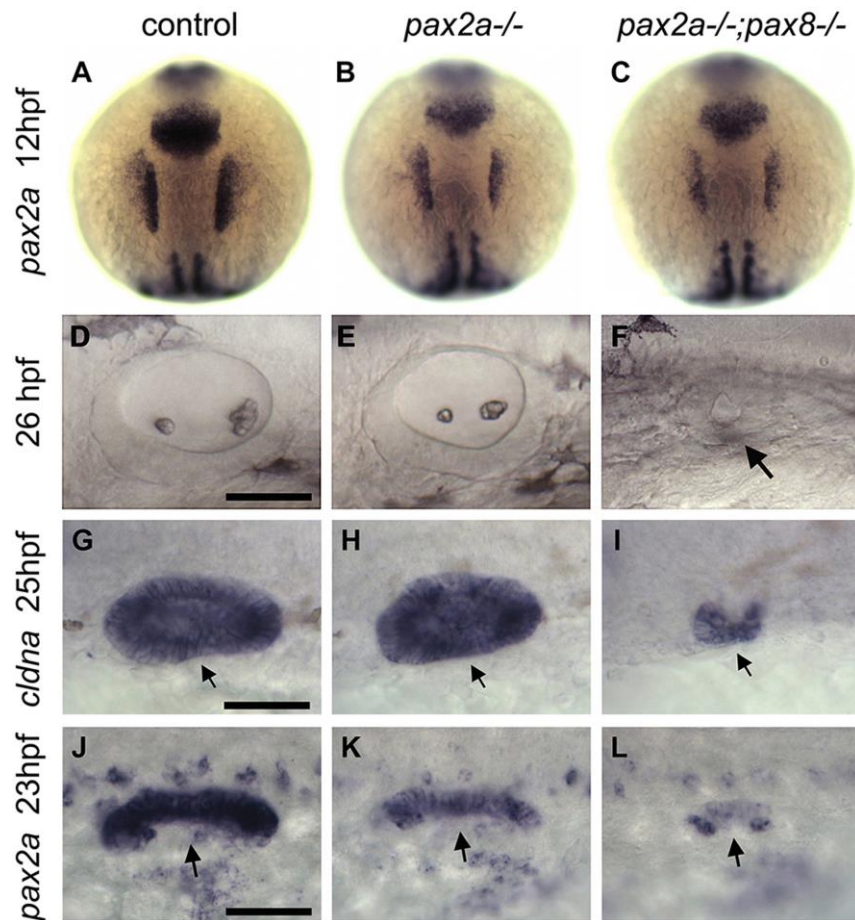
**Interactions with *pax8***

The above data show that *sox3*<sup>-/-</sup> mutants largely recapitulate our previously reported *sox3*-morphant phenotype, but we also wished to compare the phenotypes of *pax8*-morphants and *pax8*<sup>-/-</sup> mutants (Padanad and Riley, 2011). In *pax8*<sup>-/-</sup> mutants, the otic domain of *pax2a* at 12 hpf is reduced by 30% (Fig. 2.5B, D), consistent with previous morpholino data. In *pax8*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants the *pax2a* domain is reduced by 32%, which is not statistically different from *pax8*<sup>-/-</sup> mutants (Fig. 2.5C, D). This is in contrast to *pax8*-*sox3* double morphants, which show an additive reduction in the *pax2a* domain relative to *pax8*-morphants (Padanad and Riley, 2011). We also examined the interaction between *pax8* and *pax2a*. We found that *pax8*<sup>-/-</sup>; *pax2a*<sup>-/-</sup> double mutants undergo placode induction but later show severe deficiency of otic tissue, producing very small otic vesicles by 24 hpf (Fig. 2.6C, F, I, L). In contrast, injection of *pax8*-mo into *pax2a*<sup>-/-</sup> mutants usually eliminates formation of a morphological vesicle, although a small number of scattered otic cells persist (Hans et al., 2004). We draw several conclusions from these data. First, the *sox3*<sup>-/-</sup> and *pax8*<sup>-/-</sup> mutant phenotypes generally validate phenotypes produced by corresponding morpholinos when used singly, although combining these morpholinos produces an excessively severe phenotype probably reflecting additive off-target effects. Second, mutant analysis confirms that *pax8* is required to establish a normally sized placode; and *pax8* and *pax2a* are together required to maintain otic fate by the majority of otic cells. Third, mutant analysis suggests that *sox3* and *pax8* operate in the same genetic pathway, but *pax8* function is more critical.



**Figure 2.5. Interaction between *pax8*<sup>-/-</sup> and *sox3*<sup>-/-</sup> during early placode development.**

(A-C) Expression of *pax2a* at 12 hpf in a wild-type control embryo (A), a *pax8*<sup>-/-</sup> mutant (B) and a *pax8*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutant (C) embryo. Dorsal-lateral view with anterior to the left. (D) Box-and-whisker plot of relative surface area of otic/epibranchial domain of *pax2a* at 12 hpf in control, *pax8*<sup>-/-</sup> and *pax8*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutant embryos. Data are normalized relative to control groups, with means indicated by green lines. Asterisks indicate statistically significant differences relative to controls (\*\*\*)  $P < 0.001$ , Tukey's HSD test). n.s., not significantly different.



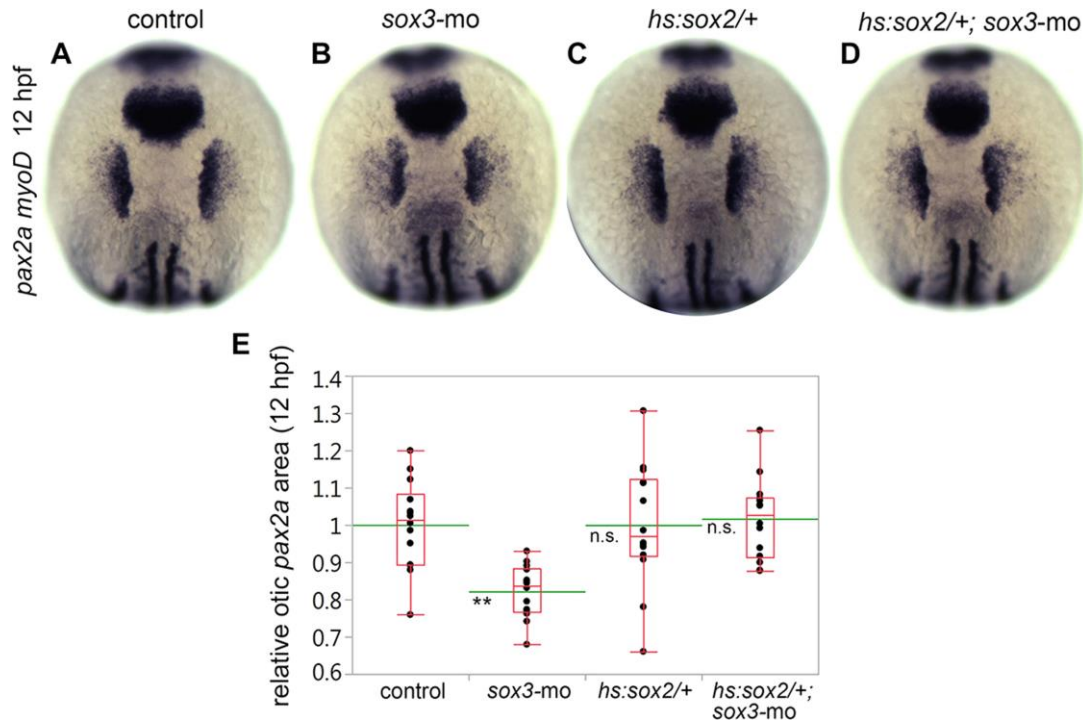
**Figure 2.6. Interaction between *pax8*<sup>-/-</sup> and *pax2a*<sup>-/-</sup> during otic development.**

(A-C) Expression of *pax2a* at 12 hpf in control (A), *pax2a*<sup>-/-</sup> (B) and *pax2a*<sup>-/-</sup>; *pax8*<sup>-/-</sup> (C) embryos (dorsal views, anterior to the top). Staging of embryos was confirmed by *myoD* expression in somites. (D-F) Lateral views (anterior to left) of the otic vesicle in control (D), *pax2a*<sup>-/-</sup> (E) and *pax2a*<sup>-/-</sup>; *pax8*<sup>-/-</sup> (F) embryos imaged live at 26 hpf. The small otic vesicle in the *pax2a*<sup>-/-</sup>; *pax8*<sup>-/-</sup> double mutant (F) is marked with an arrow. (G-L) Dorsal views (anterior to left) showing expression of *cldna* (G-I) and *pax2a* (J-L) at 25 hpf and 23 hpf respectively in control (G, J), *pax2a*<sup>-/-</sup> (H, K) and *pax2a*<sup>-/-</sup>; *pax8*<sup>-/-</sup> (I, L) embryos. Arrows indicate otic expression domains.

### Redundancy between *sox2* and *sox3*

To further address *sox2* and *sox3* function, we generated heat shock-inducible transgenes for both genes. Although *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants are similar to *sox3*<sup>-/-</sup> mutants, we reasoned that the inability of *sox2* to compensate for loss of *sox3* reflects the relatively low level

of *sox2* expression during early placodal development. To test this possibility, we injected *sox3*-mo into *hs:sox2/+* embryos to determine whether transgenic *sox2* can rescue early placodal deficiencies caused by loss of *sox3*. For this experiment we used a low level of heat shock (35 °C from 10 to 12 hpf) to avoid defects caused by high-level misexpression of *sox2* (see below). qRT-PCR analysis showed that this regimen caused a 5–6 fold increase in *sox2* transcript abundance when measured 40 min or 60 min after initiating heat shock (relative to non-transgenic *sox3*-morphants). Non-transgenic *sox3*-morphants showed an 18% reduction in the otic domain of *pax2a* at 12 hpf, similar to *sox3*<sup>-/-</sup> mutants (Fig. 2.7B, E). Weak activation of *hs:sox2* by itself had no effect on otic development but was sufficient to restore the otic domain of *pax2a* to normal in *sox3*-morphants (Fig. 2.7C, D, E). This supports the idea that *sox2* can substitute for *sox3* during early otic development.



**Figure 2.7. *sox2* can substitute for *sox3* during early otic development.**

**Figure 2.7. Continued.**

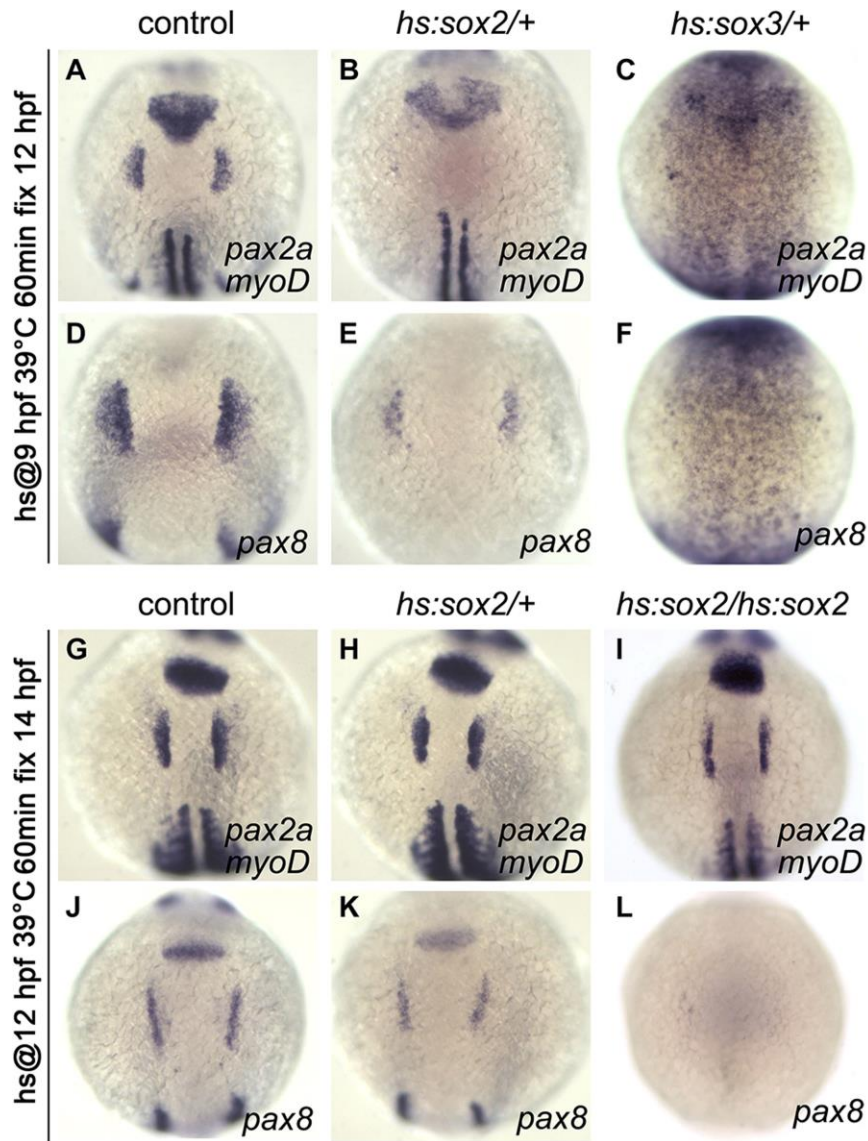
(A-D) Expression of *pax2a* at 12 hpf in a control embryo (A), a *sox3*-morphant (B), a *hs:sox2/+* heterozygote (C) and *hs:sox2/+* heterozygote injected with *sox3*-MO (D) embryo. Embryonic staging was confirmed with *myoD* expression in somites. Embryos were heat shocked at 35°C from 10-12 hpf. *sox3*-morphants in (B) and (D) were injected with 5 ng each of *sox3*-MO. (E) Box-and-whisker plots of relative surface area of the otic/epibranchial domain of *pax2a* at 12 hpf in controls, *sox3*-morphants, *hs:sox2/+* heterozygotes and *hs:sox2/+* heterozygotes injected with *sox3*-MO. Data are normalized relative to control groups, with means indicated by green lines. Asterisks indicate statistically significant differences compared to control (\*\*  $P < 0.01$ , Tukey's HSD test). n.s., no significant difference compared to controls.

**Misexpression of *sox2* and *sox3***

To further explore the functions of *sox2* and *sox3* during otic placode development, we tested the effects of misexpressing *sox2* or *sox3* at different developmental stages and expression levels using heat shock-inducible transgenes. For moderate misexpression we heat shocked *hs:sox2/+* or *hs:sox3/+* transgenic heterozygotes at 39 °C for 60 min, and for high-level misexpression we heat shocked embryos homozygous for the transgene. These regimens lead to a pulse of overexpression that peaks just after the end of the heat shock interval and decays gradually over the next several hours (Padanad et al., 2012). Moderate misexpression of *sox2* or *sox3* at 9 hpf led to strong reduction or elimination of otic expression of *pax8* and *pax2a* at 12 hpf, indicating strong repression of otic differentiation (Fig. 2.8A-F). Moderate misexpression of *sox2* at 12 hpf, after otic differentiation has already begun, did not alter otic expression of *pax2a* or *pax8* at 14 hpf (Fig. 2.8H, K). However, high-level misexpression of *sox2* at 12 hpf attenuated otic expression of *pax8* and reduced the size of the otic domain of *pax2a* at 14 hpf (Fig. 2.8I, L). In general, the effects of misexpressing *sox3* were more pronounced than *sox2*. For example, moderate misexpression of *sox3* at 12 hpf eliminated expression of *pax8* by 14 hpf and expression of *pax2a* was nearly abolished as well (data not shown). Because global misexpression of *sox2* or *sox3* could

disrupt essential signaling interactions with surrounding tissues, we generated mosaic embryos by transplanting lineage-labeled transgenic cells into unlabeled wild-type host embryos. When mosaic embryos were heat shocked at 9 hpf, transplanted *hs:sox2/+* or *hs:sox3/+* cells showed little or no otic expression of *pax8* or *pax2a* by 11 hpf, whereas surrounding wild-type cells expressed otic markers normally (Fig. 2.9A-B', E-H'). In contrast, when mosaic embryos were heat shocked at 12 hpf, transplanted *hs:sox2/hs:sox2* cells and *hs:sox3/+* cells showed normal otic expression of *pax2a* at 14 hpf (Fig. 2.9C, D, I, J). These data suggest that increasing expression of *sox2* or *sox3* at 9 hpf cell-autonomously blocks otic induction, whereas elevating *sox2* or *sox3* at 12 hpf has little effect, indicating that otic fate is stably specified.

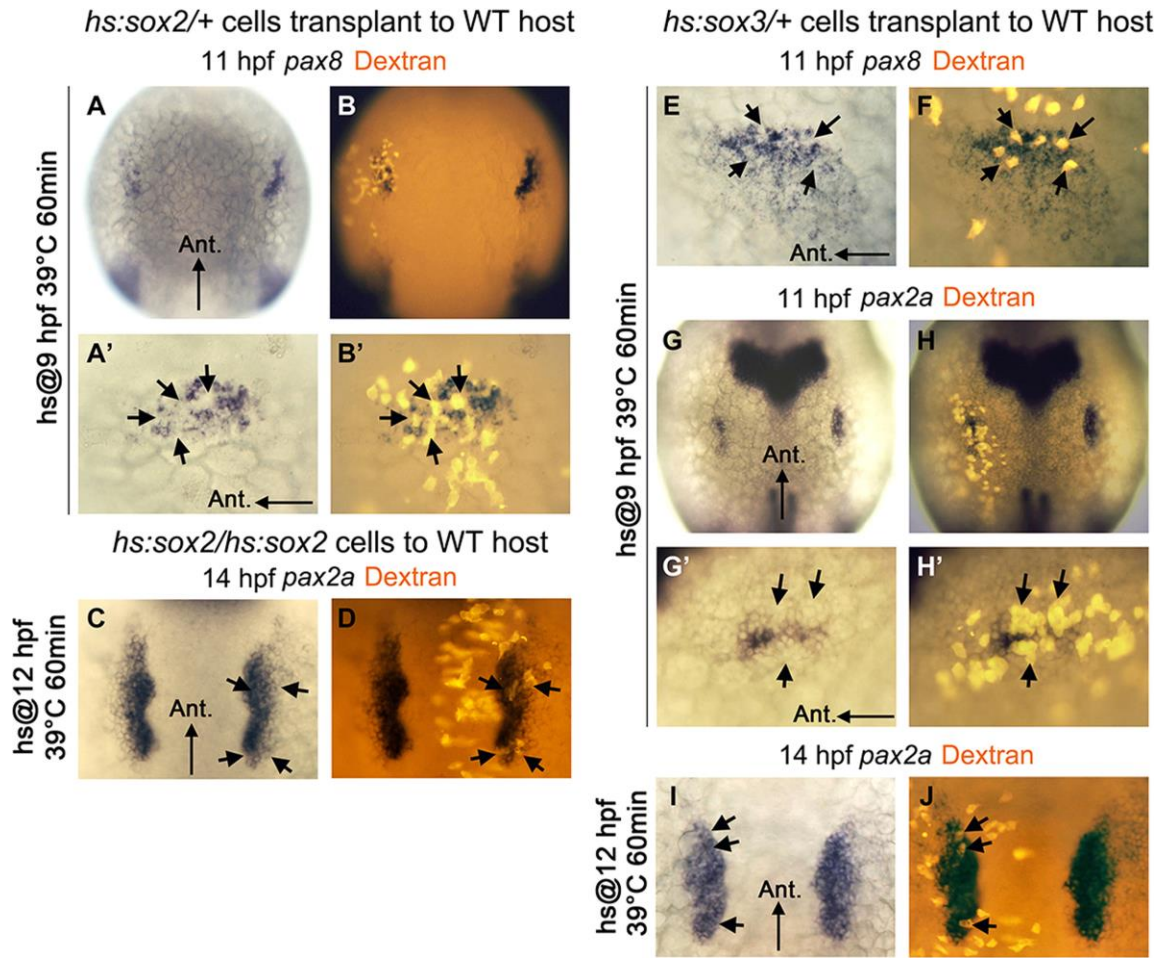




**Figure 2.8. Global misexpression of *sox2* or *sox3*.**

(A-F) Expression of *pax2a* (A-C) and *pax8* (D-F) at 12 hpf in control embryos (A, D), *hs:sox2/+* heterozygotes (B, E) and *hs:sox3/+* heterozygotes (C, F) that were heat shocked at 9 hpf, 39°C for 60 minutes. (G-L) Expression of *pax2a* (G-I) and *pax8* (J-L) at 14 hpf in control embryos (G, J), *hs:sox2/+* heterozygotes (H, K) and *hs:sox2/hs:sox2* homozygotes (I, L) that were heat shocked at 12 hpf, 39°C for 60 minutes. Embryonic staging was confirmed with *myoD* expression in somites.





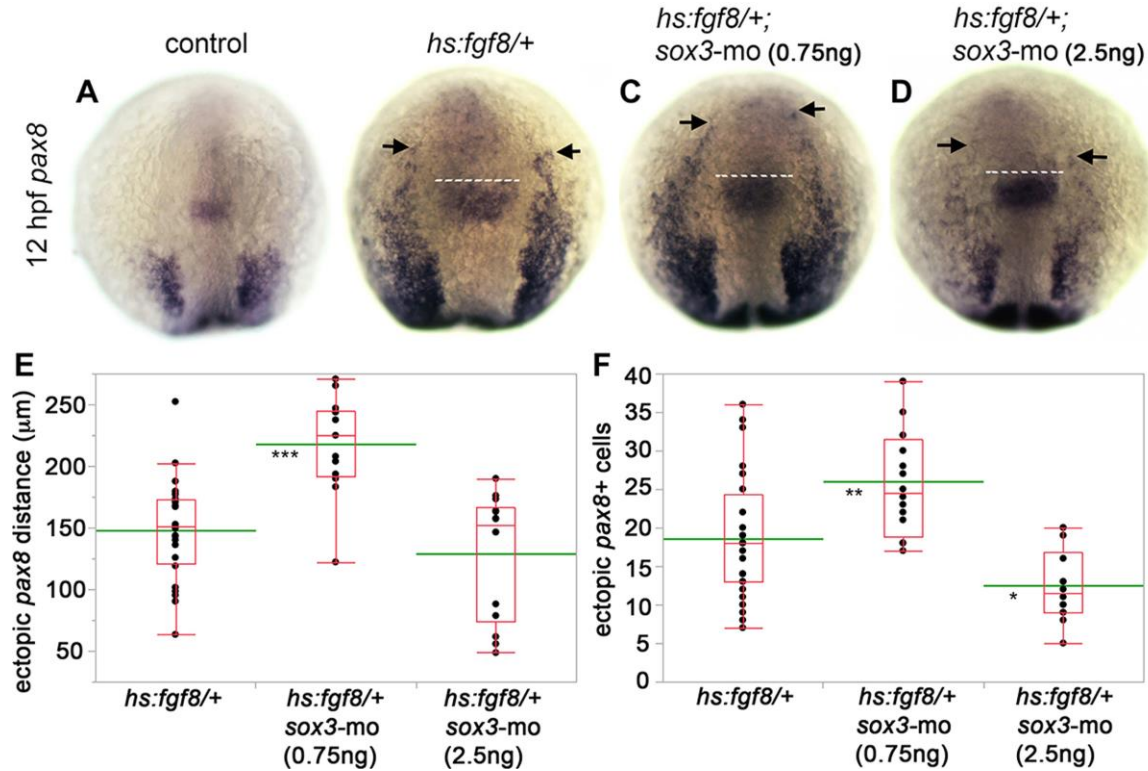
**Figure 2.9. Mosaic misexpression of *sox2* or *sox3*.**

(A-B') Expression of *pax8* at 11 hpf in a wild-type host embryo into which fluorescent dextran-labeled *hs:sox2/+* transgenic cells were transplanted. The embryo was heat shocked at 9 hpf, 39°C for 60 minutes. Bright-field (A, A') and fluorescent (B, B') images of the same specimen. An enlargement of the left otic placode (A', B') shows the positions of transgenic cells (arrows). (C, D) Bright-field (C) and fluorescent (D) images showing *pax2a* expression at 14 hpf in a wild-type host embryo with fluorescent dextran-labeled *hs:sox2/hs:sox2* transgenic cells. The embryo was heat shocked at 12 hpf, 39°C for 60 minutes. (E, F) Bright-field (E) and fluorescent (F) images showing *pax8* expression in the otic placode at 11 hpf in a wild-type host embryo with fluorescent dextran-labeled *hs:sox3/+* transgenic cells. The embryo was heat shocked at 9 hpf, 39°C for 60 minutes, and the positions of transgenic cells are indicated (arrows). (G-H') Bright-field (G, G') and fluorescent (H, H') images showing *pax2a* expression at 11 hpf in a wild-type host embryo with fluorescent dextran-labeled *hs:sox3/+* transgenic cells. The embryo was heat shocked at 9 hpf, 39°C for 60 minutes. An enlargement of the left otic placode (G', H') shows positions of transgenic cells (arrows). (I, J) Bright-field (I) and fluorescent (J) images showing *pax2a* expression at 14 hpf in a wild-type host embryo with fluorescent dextran-labeled *hs:sox3/+* transgenic cells. The embryo was heat shocked at 12 hpf, 39°C for 60 minutes. Positions of transgenic cells are indicated (arrows).

## Otic induction requires an optimal level of *sox3* expression

During normal otic induction, *sox3* is induced at a high level in the initial response to Fgf, and subsequently *sox3* expression declines to a discrete lower level in response to rising Fgf levels (Padanad and Riley, 2011). We previously reported that moderate misexpression of *fgf3* or *fgf8* at 10 hpf leads to strong ectopic expression of *sox3* throughout anterior preplacodal ectoderm, but ectopic *pax8* is not co-induced under these conditions (Padanad et al., 2012). In contrast, high-level misexpression of *fgf3* or *fgf8* leads to a reduced domain of ectopic *sox3*, as well as ectopic expression of *pax8*, *pax2a*, and other otic markers in anterior preplacodal ectoderm. We hypothesized that the high level of ectopic *sox3* induced by moderate Fgf repressed co-induction of *pax8*, whereas the lower level of *sox3* induced by high-level Fgf facilitated ectopic induction of multiple otic markers. To test this, we injected *sox3*-mo at varying concentrations into *hs:fgf8/+* embryos to titrate the level of ectopic *sox3* activity following moderate misexpression of Fgf8. When *hs:fgf8/+* embryos were injected with a low dose (0.75 ng) of *sox3*-mo and then heat shocked at 10 hpf (39 °C for 30 min), ectopic *pax8*-expressing cells were detected an average of 220  $\mu$ m anterior to the midbrain-hindbrain border, nearly to the front of the head, compared to only 148  $\mu$ m for uninjected *hs:fgf8* embryos (Fig. 2.10B, C, E). Similarly, the number of ectopic *pax8*-expressing cells produced in *hs:fgf8/+* embryos injected with 0.75 ng *sox3*-mo was on average 40% greater than in uninjected *hs:fgf8/+* embryos. Thus, partial knockdown of *sox3* enhanced the ability of *hs:fgf8* to induce ectopic otic tissue. On the other hand, injecting 2.5 ng *sox3*-mo into *hs:fgf8/+* embryos (to mimic *sox3*<sup>-/-</sup>) reduced the anterior limit of ectopic *pax8* expression to 129  $\mu$ m beyond the midbrain-hindbrain border, corresponding to 33% fewer ectopic *pax8*-expressing cells compared to uninjected *hs:fgf8/+* embryos. The level of ectopic *pax8* expression also appeared much lower in embryos injected with 2.5 ng *sox3*-mo. These data support

the hypothesis that a high level of ectopic *sox3* following moderate misexpression of Fgf hinders the ability to induce ectopic otic tissue. Furthermore, complete knockdown of *sox3* is even more detrimental to ectopic otic induction, whereas weak knockdown of *sox3* potentiates ectopic otic induction. These data are consistent with the idea that proper otic induction requires *sox3*, but the level of *sox3* must be tightly modulated to avoid suppression of differentiation.



**Figure 2.10. Ectopic otic induction requires an optimal level of *sox3*.**

(A-D) Expression of *pax8* at 12 hpf in a control (A), *hs:fgf8/+* heterozygote (B), *hs:fgf8/+* heterozygote injected with 0.75 ng *sox3*-MO (C) and *hs:fgf8/+* heterozygote injected with 2.5 ng *sox3*-MO (D) embryo. Embryos were heat shocked at 10 hpf, 39°C for 30 minutes. The anterior edge of the midbrain-hindbrain boundary (dashed white line) was used as a reference to measure ectopic expression of *pax8*. (E) Box-and-whisker plot of the distance from midbrain-hindbrain border to the anterior limit of ectopic *pax8* expression in *hs:fgf8/+* heterozygotes, *hs:fgf8/+* heterozygotes injected with 0.75 ng *sox3*-MO and *hs:fgf8/+* heterozygotes injected with 2.5 ng *sox3*-MO. (F) Box-and-whisker plot of the number (per side) of ectopic *pax8*-expressing cells anterior to the midbrain-hindbrain border in *hs:fgf8/+* heterozygotes, *hs:fgf8/+* heterozygotes injected with 0.75 ng *sox3*-MO and *hs:fgf8/+* heterozygotes injected with 2.5 ng *sox3*-MO. Green line indicates mean. Asterisks indicate statistically significant differences compared to control (\* P<0.05, \*\* P<0.01, \*\*\* P<0.001, Tukey's HSD test).

## DISCUSSION

We generated targeted knockouts and heat shock-inducible transgenic lines to evaluate the roles of *sox2* and *sox3* in development of otic and epibranchial placodes in zebrafish. Placodal expression of *sox2* had not been previously reported but we show here that its expression can be detected by early somitogenesis stages, several hours after the onset of *sox3* expression. Mutant analysis confirms that both *sox2* and *sox3* are required for normal development of otic and epibranchial placodes. Moreover, these factors show some degree of redundancy. Mild misexpression of *sox2* rescues the otic placode deficiency in *sox3*<sup>-/-</sup> mutants, and *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants show a significantly greater deficiency of epibranchial ganglia than single mutants. Although both factors are required, several observations show that the overall level of *sox2* and *sox3* expression must not exceed an upper limit during early placodal development. High-level misexpression of *sox2* or *sox3* cell-autonomously blocks initial otic induction but does not block or reverse otic fate after otic cells have begun to differentiate. Moreover, in an assay to induce ectopic otic tissue, partial knockdown of *sox3* enhances the ability of Fgf misexpression to induce ectopic *pax8* whereas full knockdown of *sox3* strongly inhibits this response. Together these data show that an optimal level of *sox2* and *sox3* are required for proper induction of otic and epibranchial placodes, with either higher or lower levels becoming inhibitory.

We also reexamined the genetic interaction between *sox3* and *pax8*. The otic placode deficiency seen in *pax8*<sup>-/-</sup> mutants is similar to *pax8*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants, suggesting that *pax8* and *sox3* act in the same genetic pathway. This is in contrast to *pax8-sox3* double morphants,

which show an additive deficiency of otic tissue. Thus, while *sox3*<sup>-/-</sup> and *pax8*<sup>-/-</sup> morphant phenotypes closely resemble corresponding mutant phenotypes, the double morphant phenotype appears too severe and probably reflects additive off-target effects.

### **A common Pax2/8-Sox2/3 pathway**

How do Pax8 and Sox3 work together to promote otic induction? One possibility is that Pax8 and Sox3 bind independently to a common set of enhancers of early otic genes, with both factors being required for full activation. Alternatively, Sox3 and Pax8 could form heterodimers required for enhancer binding. An analogous situation has been documented during development of the lens placode, in which Sox2 and Pax6 dimerize to bind the DC5 lens enhancer (Kamachi et al., 2001). Binding is cooperative such that loss of either factor severely attenuates transcription. This accounts for why loss of either Sox2 or Pax6 blocks lens placode development at a very early stage (Smith et al., 2009). A similar Sox2-Pax6 partnership maintains neural progenitors in the olfactory (Guo et al., 2010; Packard et al., 2016), although only Pax6 is required to establish the olfactory placode (Hogan et al., 1988). By comparison, induction of otic/epibranchial placodes is less critically dependent on *sox3* and *pax8*, partly reflecting greater genetic redundancy in otic/epibranchial placodes. Disruption of both *pax8* and *pax2a* leads to gradual loss of most otic cells by 24 hpf (Fig. 2.5, and Hans et al., 2004), and simultaneous knockdown of a third homolog *pax2b* leads to loss of all otic cells through dedifferentiation (Mackereth et al., 2005). Disruption of *sox2* and *sox3* has much milder effects on placode development, perhaps similar to the role of Sox2 in the olfactory epithelium but marking a distinct difference from the lens placode.

### **Redundancy and compensation between *sox2* and *sox3***

Although zebrafish *sox3*<sup>-/-</sup> mutants are viable and fertile, it is noteworthy that these mutants show roughly 2-fold upregulation of *sox2* (Fig. 2.1), which could compensate for loss of *sox3*. Such compensation does not ameliorate the early otic deficiency in *sox3*<sup>-/-</sup> mutants since simultaneous loss of *sox2* does not worsen the phenotype. Nevertheless, compensation from *sox2* could facilitate restoration of normal otic development during later stages of development in *sox3*<sup>-/-</sup> mutants. Interestingly, *Sox3* knockout mice are also viable, but in this case there is no detectable upregulation of *Sox2* (Adikusuma et al., 2017). Otic development is expected to be normal in *Sox3* mutants since *Sox3* is not detectably expressed in otic cells in mouse. Of the three *SoxB1* genes in mouse, only *Sox2* is expressed during early otic cells by the otic placode/otic cup stage. Unfortunately, conditional knockouts of mouse *Sox2* have not been conducted early enough to evaluate potential deficiencies in otic placode formation. On the other hand, knockouts induced during early otic vesicle stage block development of neurons and sensory epithelia (Kiernan et al., 2005; Steevens et al., 2017). At later stages in zebrafish, *sox2* and *sox3* act non-redundantly to regulate development of sensory epithelia and neurons, respectively (Gou et al., 2018).

### **Sensitivity to Sox2/Sox3 concentration**

There are two general mechanisms by which Sox2/Sox3 concentration critically affects functional output. First, Sox2 has been shown to act as a “pioneer factor” that can open large tracts of condensed chromatin (Soufi et al., 2015). Sox2 has the unusual ability to recognize specific and non-specific sequences on DNA wrapped around nucleosomes, as well as distinct consensus sites within open DNA. Binding to these sites is differentially sensitive to concentration. Therefore, elevating Sox2 levels could open larger sized tracts of chromatin and bind a wider array of

sequences, potentially activating expression of repressors that antagonize normal otic development.

Second, SoxB1 function is often modified by interacting with other cofactors, with functional output being highly sensitive to modest changes in concentration (Kondoh and Kamachi, 2010; Rizzino and Wuebben, 2016). In embryonic stem cells, for example, the concentration of Sox2 and Oct4 must be maintained within a narrow range to maintain pluripotency (Rizzino and Wuebben, 2016). Sox2 and Oct4 bind cooperatively into multi-subunit complexes that activate transcription of their own genes, as well as other pluripotency genes (Ambrosetti et al., 1997; Boer et al., 2007; Chew et al., 2005). In this way, Sox2 and Oct4 establish a self-reinforcing gene regulatory network that maintains pluripotency. However, when Sox2 levels are further elevated, its occupancy on relevant enhancers increases and leads to repression of all genes in the network (Boer et al., 2007; Kopp et al., 2008). This constitutes a binary switch that, once tripped, inhibits pluripotency and initiates differentiation. The mechanism of Sox2's repressor activity is unknown but requires its C-terminal transactivation domain (Boer et al., 2007). Though historically associated with activation, this domain can also bind transcriptional repressors such as HDAC1 and HDAC2 (Cox et al., 2010).

Sox2 levels are also critical for tissue-progenitors during later stages of development, but the dose-response is opposite to that seen in embryonic stem cells. Specifically, high levels of Sox2 are required to maintain pluripotency of tissue-specific progenitors whereas lower levels promote differentiation (Gómez-López et al., 2011, Hutton and Pevny, 2011, Juuri et al., 2012, Matsushima et al., 2011, Packard et al., 2016, Tucker et al., 2010). The switch to differentiation involves

changes in Sox2-partner binding (Kondoh and Kamachi, 2010), but how this relates to Sox2 concentration remains unknown.

There has been much less molecular analysis of Sox3 but it is believed to function in tissue-progenitors in a manner similar to Sox2 (Stavridis et al., 2007; Wang et al., 2006). Indeed, conditional knockout of Sox2 in mouse leads to compensatory upregulation of Sox3, which is thought to ameliorate the phenotype (Miyagi et al., 2008). ChIP-Seq experiments show that Sox3 binds thousands of sites in neural progenitors, many of which are associated with genes known to regulate neural development (McAninch and Thomas, 2014). Additionally, overexpression of Sox3 maintains neural progenitors in a cycling state and blocks their differentiation (Bylund et al., 2003; Lee et al., 2012). In the zebrafish otic placode, *sox3* is initially expressed at a high level (Nikaido et al., 2007; Sun et al., 2007) and later downregulates to a discrete lower level in response to local elevation of Fgf (Padanad and Riley, 2011). The decline in *sox3* expression occurs by 12 hpf, but this is after otic fate is stably specified (Fig. 2.9) and is therefore not required for initiating or stabilizing otic induction. However, adjusting expression levels of *sox2* and *sox3* during early placodal development is required for proper development of sensory and neurogenic domains that form later within the floor of the otic vesicle (Gou et al., 2018).



## CHAPTER III

# SOX2 AND SOX3 PLAY UNIQUE ROLES IN DEVELOPMENT OF HAIR CELLS AND NEURONS IN THE ZEBRAFISH INNER EAR\*\*

## INTRODUCTION

Development of the inner ear is a highly dynamic process in which neurons, sensory epithelia, and a variety of non-sensory cell types arise from a simple epithelial structure, the otic placode. The otic placode quickly forms a fluid-filled cyst, the otic vesicle, which then further elaborates the complex shape and array of cell types comprising the inner ear. Sensory epithelia and neural progenitors originate in a ventral region of the early otic vesicle. In mammals and birds, neuroblasts arise first and subsequently delaminate from the otic epithelium and are quickly replaced by developing sensory epithelia (Raft and Groves, 2015; Raft et al., 2007). In zebrafish, neuroblasts and sensory epithelia initially form simultaneously in abutting domains in the floor of the otic vesicle, after which neuroblasts delaminate and differentiate in a manner similar to tetrapod vertebrates (Haddon and Lewis, 1996, Kantarci et al., 2016, Millimaki et al., 2007). Despite differences in timing and degree of spatial overlap of neural vs. sensory development, many of the same regulatory genes operate in all vertebrate species. The transition from neural to sensory development in mammals is triggered in part by cross-repression between proneural and prosensory factors *Ngn1* and *Atoh1* (Raft et al., 2007). In principle a similar cross-repression could help stabilize spatial segregation of sensory and neural fates in zebrafish, although this has not been formally investigated.

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In all vertebrates, members of the SoxB1 family of transcription factors are required for normal development of both sensory epithelia and neurons of the inner ear (reviewed by Raft and Groves, 2015). In non-mammalian vertebrate species, *Sox3* is the first to be expressed during placodal development and appears to presage neural development, marked by expression of the proneural gene *Neurog1* (Abello et al., 2010; Neves et al., 2007; Nikaido et al., 2007; Sun et al., 2007). *Sox2* is expressed at later stages and is associated with development of sensory epithelia, marked by expression of the prosensory gene *Atoh1* (Millimaki et al., 2010; Neves et al., 2007). However, *Sox2* and *Sox3* show overlapping domains of expression for extended periods of otic development. This raises questions about whether *Sox2* and *Sox3* act redundantly in otic development, and how their cell-type specific functions are regulated (i.e. how do they differentially activate *Neurog1* vs. *Atoh1*). In mammals, *Sox3* is not expressed in the otic vesicle, whereas *Sox2* is expressed in the floor of the otic vesicle and is required for both neurons and sensory epithelia (Kiernan et al., 2005; Puligilla et al., 2010; Steevens et al., 2017). Moreover, replacement of the *Sox3* coding region with *Sox2* permits development of mice that are viable and morphologically normal, suggesting that *Sox2* and *Sox3* are largely redundant (Adikusuma et al., 2017). Nevertheless, it is still not understood how *Sox2* can regulate both neurons and sensory epithelia in the same tissue, a problem that is presumably related to how *Sox2* and *Sox3* regulate different cell fates in non-mammalian vertebrates.

SoxB1 factors are well known for providing two seemingly contradictory functions. They can maintain pluripotency of stem cells and progenitors (Bylund et al., 2003; Goldsmith et al., 2016; Rizzino and Wuebben, 2016; Surzenko et al., 2013; Tucker et al., 2010), and they can promote early differentiation of various cell types and tissues (Amador-Arjona et al., 2015; Archer

et al., 2011; Hoffmann et al., 2014; Okuda et al., 2010; Rogers et al., 2009). A number of studies have shown that the nature of SoxB1 function can vary depending on their level of expression (Boer et al., 2007, Hutton and Pevny, 2011, Kopp et al., 2008, Rizzino and Wuebben, 2016) or the availability of region- or stage-specific cofactors (Ambrosetti et al., 1997, Boer et al., 2007, Chew et al., 2005, Kamachi et al., 2001, Kondoh and Kamachi, 2010). It is unknown which of these variables regulates the ability of Sox2 and Sox3 (or Sox2 alone) to differentially regulate neural and sensory fates in the otic vesicle.

We have investigated the shared and unique functions of *sox2* and *sox3* in zebrafish through analysis of knockout lines and heat shock-inducible transgenes. Mutant analysis confirms that *sox2* is uniquely required for normal sensory development whereas *sox3* is uniquely required for neural development. Some misexpression studies also support these gene-specific functions. For example, misexpression of *sox2* at a moderate level during placodal development expands the domain of sensory development in the otic vesicle while restricting the domain of neurogenesis. However, when misexpressed at high levels, *sox2* and *sox3* mimic each other and lead to dramatic expansion of sensory and neural fates throughout the medial and lateral walls of the otic vesicle, respectively. The ability to expand sensory fates, but not neural fate, requires the medial factor *pax2a*. Early misexpression of *sox2* or *sox3* did not accelerate the onset of sensory or neural development, rather expansion of these fates occurred gradually after formation of the otic vesicle. Moreover, misexpression of *sox2* or *sox3* at later stages temporarily halts expression of prosensory and proneural factors *atoh1a* and *neurog1*. Analysis of additional markers shows that misexpression of *sox2* or *sox3* expands anterior-ventral identity (including the zones of sensory-neural competence) throughout the otic vesicle. Together these data suggest that *sox2* and *sox3*

promote sensory-neural competence while delaying onset of sensory and neural differentiation. Regionally expressed factors such as *pax2a* and *fgf8* then help to diversify *sox2/3* function to establish sensory and neural fates in spatially segregated domains. We also confirm that cross-repression between *atoh1a* and *neurog1* help reinforce the sensory-neural boundary.

## MATERIALS AND METHODS

### Fish strains and developmental conditions

Wild-type zebrafish were derived from the AB line (Eugene, OR). Mutant alleles *sox2*<sup>x50</sup>, *sox3*<sup>x52</sup> (Gou et al., 2018) and *mib*<sup>ta52b</sup> (Itoh et al., 2003; Jiang et al., 1996) and genotyping methods were previously described. Transgenic line *TG(brn3c:gap43-GFP)* (Xiao et al., 2005) was used to visualize sensory hair cells. Transgenic lines *TG(hsp70:fgf8a)*<sup>x17</sup>, *TG(hsp70:atoh1a)*<sup>x20</sup>, *TG(hsp70:sox2)*<sup>x21</sup> (Millimaki et al., 2010), *TG(hsp70:sox3)*<sup>x32</sup> (Gou et al., 2018) and *TG(hsp70:ngn1)*<sup>x28</sup> (Kantarci et al., 2016) used in the misexpression studies here were referred to as *hs:fgf8*, *hs:atoh1a*, *hs:sox2*, *hs:sox3* and *hs:neurog1* respectively. Embryos were developed under standard conditions at 28.5 °C (Kimmel et al., 1995), except during and after heat shock, in fish water containing methylene blue. Embryos were staged based on standard morphological features (Kimmel et al., 1995). To prevent melanin formation in older embryos (>24 hpf), PTU (1-phenyl 2-thiourea, 0.3 mg/ml) was added to fish water during development.

## Gene misexpression and morpholino injections

Misexpression of various genes was achieved by briefly incubating embryos heterozygous or homozygous for heat shock inducible transgenes in a water bath at 38 or 39 °C for 30 or 60 min, except where noted in the text. For experiments involving activation of *hs:sox3* at 12 hpf, heat shock was performed at 38 °C because 39 °C heat shock at this time led to severe axial truncation by 24 hpf, precluding meaningful interpretation of results. In contrast, activation of *hs:sox3* at 39 °C was readily tolerated at later stages. In all cases, after heat-shock embryos were maintained at 33 °C until fixation. Wild-type embryos were also heat shocked to serve as controls for all misexpression studies. Knock-down of *pax2a* was achieved by injecting 5 ng of morpholino oligomers (mo), obtained from Gene Tools, Inc., into one-cell stage embryos. Sequence of *pax2a*-mo was previously described (Bricaud and Collazo, 2006). In all morpholino knock-downs, embryos were co-injected with *p53*-mo (Robu et al., 2007) to prevent non-specific cell death. Phenotypes described here were assessed in at least 15 embryos per probe and time point unless stated otherwise.

## In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization, two-color in situ hybridization and immunostaining were performed as previously described (Jowett and Yan, 1996; Phillips et al., 2001; Riley et al., 1999). Primary antibody used to label mature neurons of statoacoustic ganglion (SAG) is anti-Islet1/2 (Developmental Studies Hybridoma Bank 39.4D5, 1:100), secondary antibody is Alexa 546 goat anti-mouse IgG (ThermoFisher Scientific A-11003, 1:50). For cell proliferation analysis, anti-phospho-Histone H3 (EMD MILLIPORE 06-570, 1:350) and Alexa 546 goat anti-rabbit IgG (ThermoFisher Scientific A-11010, 1:50) were used.

## Cell transplantation and cryo-sectioning

Donor *hs:sox3/hs:sox3* embryos were injected with lineage tracer (10,000 MW, lysine-fixable tetramethylrhodamine labeled dextran in 0.2 M KCl) at one-cell stage. Donor cells were transplanted into unlabeled wild-type host embryos during late blastula stage. Embryos stained by whole-mount in situ hybridization were processed for cryo-sectioning as previously described (Vemaraju et al., 2012) and cut into serial 10- $\mu$ m sections then mounted in 30% glycerol, except that sections from mosaic embryos were mounted in SlowFade Gold antifade reagent with DAPI (Life technologies).

## Genotyping and data analysis

To identify *sox2*<sup>-/-</sup>, *sox3*<sup>-/-</sup> single mutants and *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants from *sox2*<sup>+/-</sup>; *sox3*<sup>+/-</sup>; *brn3c:gap43-GFP* triple carrier intercross, tails of individual embryos, post Islet1/2 immunostaining, were used for DNA extraction and single-embryo genotyping described previously (Gou et al., 2018). Quantification of gene expression area was performed using Photoshop measuring number of pixels. Areas shown in the figures were normalized relative to wild-type control embryos. Quantification of number of cells expressing certain gene or protein was done in either whole mounts (*atoh1b*, phospho-Histone H3 and Islet1/2 staining) or in serial sections (*atoh1a*, *neurog1* staining in mosaic embryos). Hair cells were counted in fixed whole mount embryos by imaging *TG(brn3c:gap43-GFP)* fluorescence, a stable marker of mature hair cells (Xiao et al., 2005). Mature SAG neurons were counted in whole mounts stained with anti-Islet1/2 monoclonal antibody. Expression of Islet1/2 marks SAG cells that have completed migration, become post-mitotic, and sprouted projections to synaptic targets (Vemaraju et al., 2012).

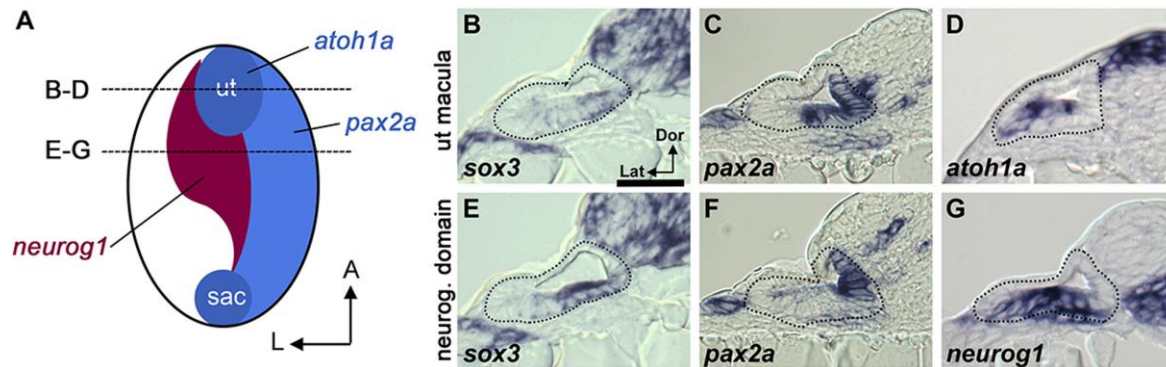
Statistical pair-wise comparisons were performed using students' t-test. For experiments that involve more than two groups, significance was evaluated by ANOVA and Tukey's post-hoc HSD tests.

## RESULTS

### Expression of *sox3* in the otic vesicle

Although expression of *sox3* is a well-known marker of early development of the otic placode (Nikaido et al., 2007; Sun et al., 2007), expression in the otic vesicle has not been described. We therefore characterized expression of *sox3* in the otic vesicle at 24 hpf with respect to *neurog1* in the neurogenic domain, *atoh1a* in sensory maculae, and the medial marker *pax2a* (Fig. 3.1A). Cross sections through the utricular macula show that *sox3* is expressed at a relatively low level and overlaps with both *atoh1a* and *pax2a* (Fig. 3.1B–D). Slightly more posterior sections passing through the widest part of the neurogenic domain show that *sox3* overlaps extensively with *neurog1*, but neither gene overlaps with *pax2a* (Fig. 3.1E–G). Additionally, *sox3* is expressed in a gradient, with high levels in the medial half of the neurogenic domain and falling rapidly towards the lateral half. The lateral domain corresponds to the region where neuroblasts delaminate, a function that requires overlap of *neurog1* with a lateral domain of *gooseoid* (*gsc*) expression (Kantarci et al., 2016). Thus, *sox3* is widely expressed in the floor of the otic vesicle but shows highest expression in newly specified neuroblasts, with levels declining as neuroblasts mature and delaminate. Additionally, the sensory-neural boundary corresponds closely to the *pax2a*

expression domain. We previously reported that *sox2* expression is restricted to prosensory regions of the otic placode and vesicle (Millimaki et al., 2010) and that sensory epithelia coexpress and upregulate *pax2a* (Riley et al., 1999).



**Figure 3.1. Expression of *sox3* in the otic vesicle overlaps sensory and neurogenic domains.** (A) Schematic depiction of the floor of the otic vesicle (anterior up, lateral to the left) showing expression domains of *atoh1a* in the utricular (ut) and saccular (sac) maculae, *neurog1* in the neurogenic domain, and the medial marker *pax2a*. Dashed lines indicate the section planes shown in (B-D) and (E-G). (B-D) Expression of *sox3* (B), *pax2a* (C) and *atoh1a* (D) in cross sections passing through the utricular macula at 24 hpf. (E-G) Expression of *sox3* (E), *pax2a* (F) and *neurog1* (G) in cross sections passing through the widest part of the neurogenic domain at 24 hpf. Otic vesicle borders are outlined in B-G.

### Requirements for *sox2* and *sox3* in sensory and neural development

To test whether *sox2* and *sox3* play redundant or distinct roles in otic development, we examined accumulation of hair cells and neurons of the statoacoustic ganglion (SAG) in embryos disrupted for *sox2* or *sox3* or both *sox2* and *sox3*. *sox2*<sup>-/-</sup> mutants show a 25% reduction in the number of hair cells at 38.5 hpf (Fig. 3.2A) and often display 1–2 dying hair cells being extruded from the otic epithelium, similar to previous findings in *sox2* morphants (Millimaki et al., 2010). In contrast, the number of mature SAG neurons at 36 hpf is normal in *sox2*<sup>-/-</sup> mutants (Fig. 3.2B). Conversely, *sox3*<sup>-/-</sup> mutants produce a normal number of hair cells (Fig. 3.2A) but the number of

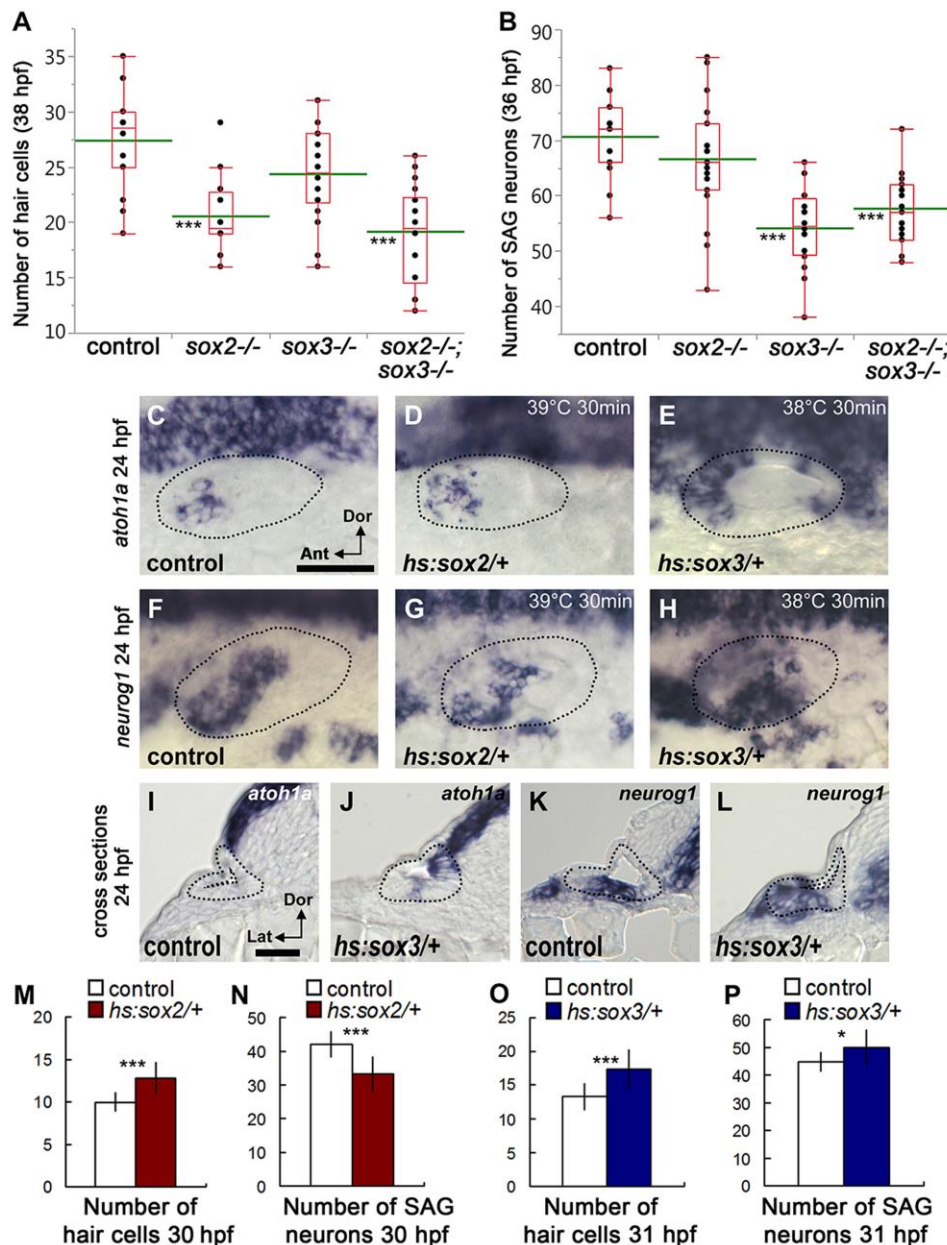


mature SAG neurons is reduced by 23% at 36 hpf (Fig. 3.2B). Interestingly, *sox2*<sup>-/-</sup>;*sox3*<sup>-/-</sup> double mutants do not show additional reduction in accumulation of hair cells or SAG neurons compared to *sox2*<sup>-/-</sup> and *sox3*<sup>-/-</sup> single mutants, respectively (Fig. 3.2A, B). These data show that *sox2* and *sox3* are uniquely required for sensory and neural development, respectively, and there is no synergistic interaction between *sox2* and *sox3*.

### **Misexpression of *sox2* and *sox3***

To further explore the unique and overlapping functions of *sox2* and *sox3*, we tested the effects of misexpressing *sox2* or *sox3* at different developmental stages and with varied expression levels using heat shock-inducible transgenes. We began by misexpressing *sox2* or *sox3* at 12.5 hpf (7 somites stage), when developing otic cells are still uncommitted. For moderate misexpression, transgenic *hs:sox2*<sup>+</sup> and *hs:sox3*<sup>+</sup> heterozygotes were heat shocked for 30 min at 38–39 °C (see Materials and Methods), yielding a pulse of transgene activity lasting roughly 2–3 h after the end of the heat shock period (Padanad et al., 2012). Moderate misexpression of *sox2* at 12.5 hpf led to a modest expansion of sensory epithelia at 24 hpf as shown by a slightly expanded domain of *atoh1a* (Fig. 3.2D) and a 29% increase in mature hair cells by 30 hpf (Fig. 3.2M). Under these same conditions, there was a marked reduction in the neurogenic domain at 24 hpf marked by *neurog1* (Fig. 3.2G) and the number of mature SAG neuron was reduced by 21% at 30 hpf (Fig. 3.2N). In contrast to *sox2*, moderate misexpression of *sox3* expanded both sensory and neurogenic domains at 24 hpf (Fig. 3.2E, H). Sections through the middle of the otic vesicle revealed ectopic *atoh1a* expressing cells in the medial wall of the otic vesicle (Fig. 3.2J compare to 3.2I), while ectopic *neurog1* expressing cells appeared in the lateral wall of the otic vesicle (Fig. 3.2L compare to 3.2K). At later stages there was a corresponding increase in the number of mature hair cells and

SAG neurons (Fig. 3.2O, P). Thus, when misexpressed at moderate levels, *sox2* promotes sensory development and impairs neurogenesis, whereas *sox3* promotes both sensory and neural development.



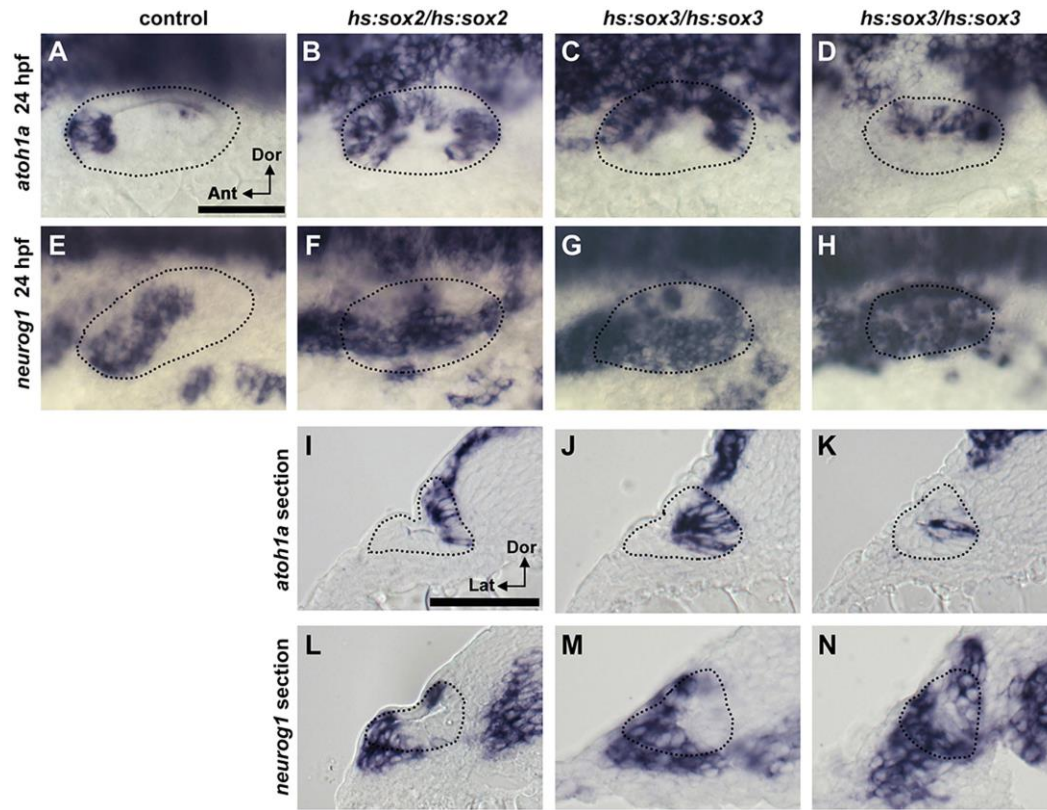
**Figure 3.2. Distinct roles for *sox2* and *sox3* in sensory and neural development.**

(A, B) Box-and-whisker plots of the total number of hair cells at 38 hpf (A) and mature SAG neurons at 36 hpf (B) in control, *sox2*<sup>-/-</sup>, *sox3*<sup>-/-</sup> and *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutant embryos.

**Figure 3.2. Continued.**

Green lines represent means. Asterisks indicate statistically significant differences compared to controls (\*\*\* $P < 0.001$ , Tukey's HSD test following ANOVA). (C-H) Dorsolateral views (anterior to left) of expression of *atoh1a* (C-E) and *neurog1* (F-H) at 24 hpf in control embryos, *hs:sox2/+* heterozygotes and *hs:sox3/+* heterozygotes. Embryos were heat shocked at 12.5 hpf, 38°C or 39°C for 30 minutes, as indicated. (I-L) Expression of *atoh1a* (I, J) and *neurog1* (K, L) at 24 hpf in cross sections through the middle of the otic vesicle in control embryos (I, K) and *hs:sox3/+* heterozygotes (J, L). Embryos were heat shocked at 12.5 hpf, 38°C for 30 minutes. Otic vesicle borders are outlined in C-L. (M, N) Quantification of the total number of hair cells (M) and mature SAG neurons (N) at 30 hpf in control and *hs:sox2/+* embryos. (O, P) Quantification of the total number of hair cells (O) and mature SAG neurons (P) at 31 hpf in control and *hs:sox3/+* embryos. Error bars represent standard deviation in M-P, and asterisks indicate statistically significant differences relative to controls (\* $P < 0.05$ , \*\*\* $P < 0.001$ , student's t-test,  $n > 13$ ).

To achieve higher levels of misexpression, we increased transgene copy-number by generating *hs:sox2/hs:sox2* and *hs:sox3/hs:sox3* homozygotes. High-level misexpression of *sox2* at 12.5 hpf led to a dramatic expansion of both sensory (Fig. 3.3B compare to 3.3A) and neurogenic domains by 24 hpf (Fig. 3.3F compare to 3.3E), with ectopic *atoh1a* expressing cells filling the medial wall (Fig. 3.3I) and some ectopic *neurog1* expressing cells spreading into the lateral wall (Fig. 3.3L). High-level misexpression of *sox3* at 12.5 hpf usually led to similar but even more pronounced expansion of sensory and neurogenic domains at 24 hpf (Fig. 3.3C, G), such that almost all medial cells express *atoh1a* and almost all lateral cells express *neurog1* (Fig. 3.3J, M). However, in a subset of *hs:sox3/hs:sox3* embryos, the neurogenic domain expanded to encompass nearly the entire otic vesicle including the medial wall (Fig. 3.3H, N) with a corresponding contraction of the sensory domain (Fig. 3.3D, K). Thus, when expressed at high levels, *sox2* and *sox3* can greatly expand both sensory and neural fates, and in extreme cases *sox3* can lead to acquisition of neural fate by nearly all otic cells.



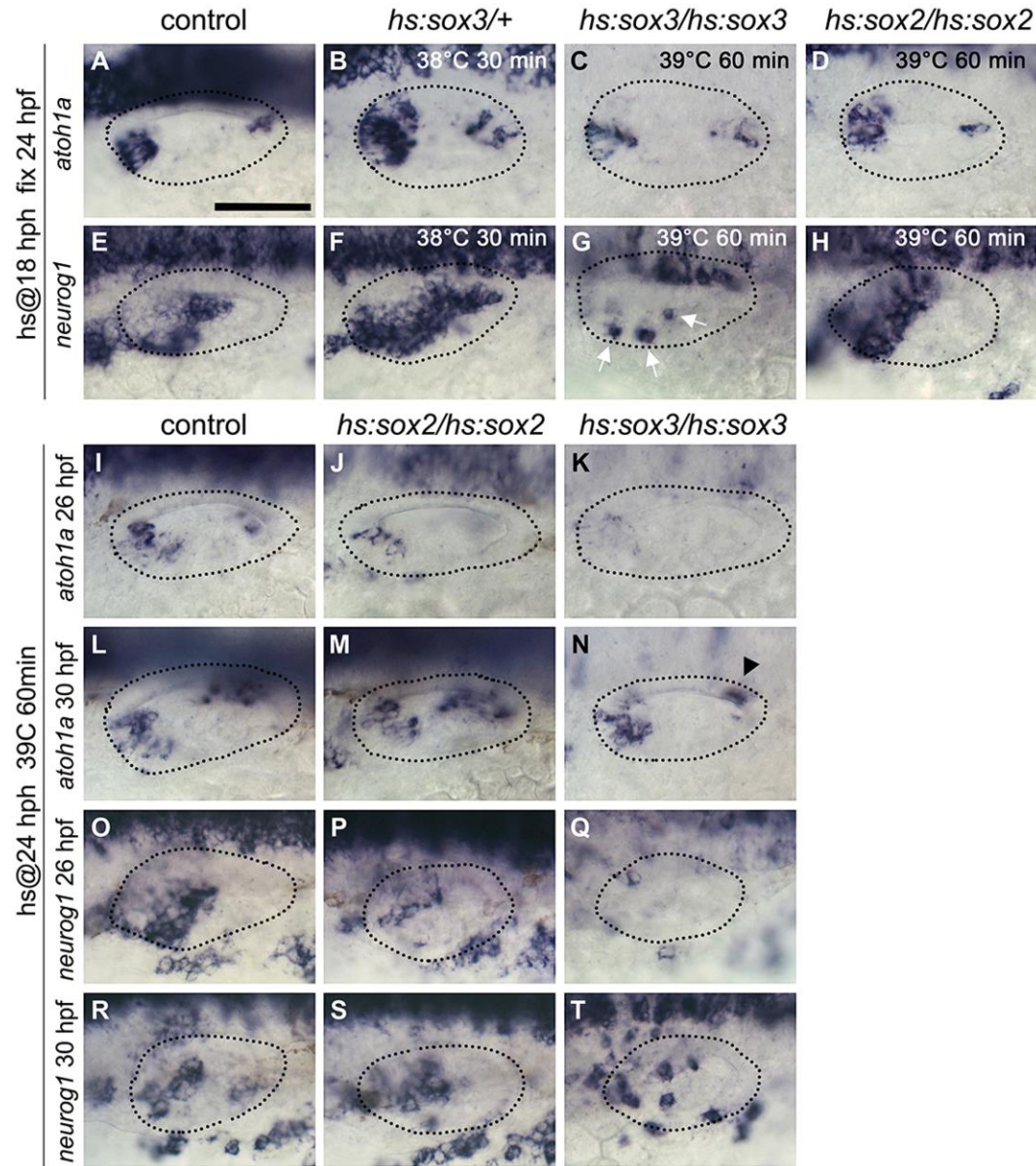
**Figure 3.3. Effects of high-level misexpression of *sox2* or *sox3* during early placode development.**

(A-H) Dorsolateral views (anterior to the left) of expression of *atoh1a* (A-D) and *neurog1* (E-H) at 24 hpf in control (A, E), *hs:sox2/hs:sox2* (B, F) and *hs:sox3/hs:sox3* (C-H) embryos. (I-N) Expression of *atoh1a* (I-K) and *neurog1* (L-N) at 24 hpf in cross sections (lateral to the left) through the middle of the otic vesicle in *hs:sox2/hs:sox2* homozygotes (I, L) and *hs:sox3/hs:sox3* homozygotes (J, K, M, N). *hs:sox2/hs:sox2* homozygotes were heat shocked at 12.5 hpf, 39°C for 60 minutes, whereas *hs:sox3/hs:sox3* homozygotes were heat shocked at 12.5 hpf, 38°C for 30 minutes. Otic vesicle borders are outlined.

We next tested the effects of misexpressing *sox2* or *sox3* at later stages of otic development. Moderate misexpression of *sox2* at 18 hpf caused no obvious changes in expression of *atoh1a* or *neurog1* at 24 hpf (data not shown), and high-level misexpression of *sox2* at 18 hpf caused only a modest increase in sensory and neurogenic domains at 24 hpf (Fig. 3.4D, H). Moderate misexpression of *sox3* at 18 hpf also led to a modest expansion of sensory and neurogenic domains



at 24 hpf (Fig. 3.4B, F). In contrast, high-level misexpression of *sox3* at 18 hpf strongly reduced expression of *atoh1a* and *neurog1* at 24 hpf (Fig. 3.4C, G), indicating suppression of sensory and neural development.



**Figure 3.4. Effects of misexpressing *sox2* or *sox3* at later stages.**

(A-H) Expression of *atoh1a* (A-D) and *neurog1* (E-H) at 24 hpf in control (A, E), *hs:sox3/+* (B, F), *hs:sox3/hs:sox3* (C, G) and *hs:sox2/hs:sox2* (D, H) embryos. Embryos were heat-shocked at 18 hpf with varying temperatures and durations as indicated. White arrows indicate otic expression of *neurog1* in G. (I-N) Expression of *atoh1a* at 26 hpf (I-K) and 30 hpf (L-N) in control (I, L), *hs:sox2/hs:sox2* (J, M) and *hs:sox3/hs:sox3* (K, N) embryos following heat-shock

**Figure 3.4. Continued.**

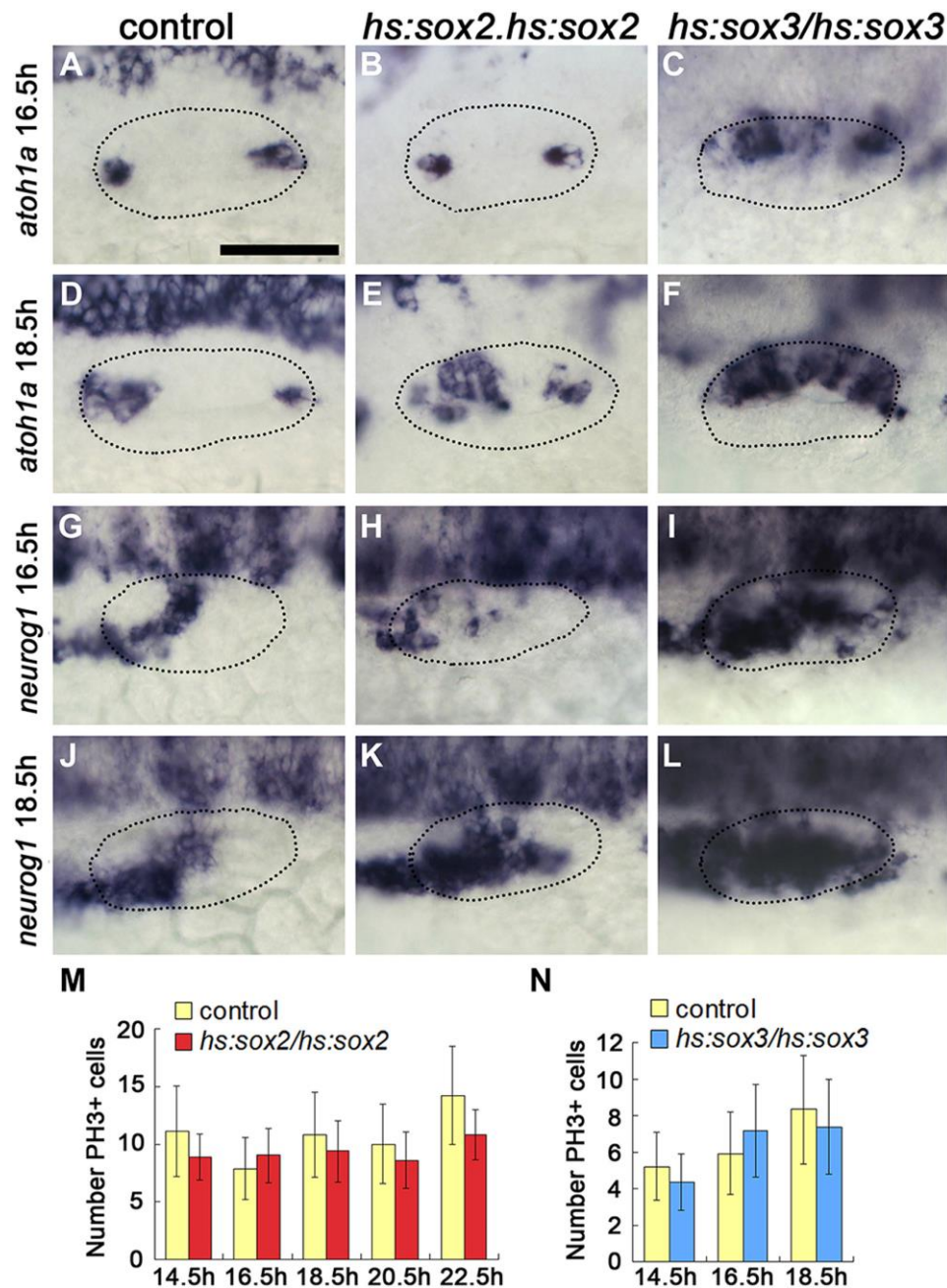
(39°C for 60 minutes) at 24 hpf. Black arrowhead indicates *atoh1a* expression in the saccular macula in N, which is strongly reduced compare to L. (O-T) Expression of *neurog1* at 26 hpf (O-Q) and 30 hpf (R-T) in control (O, R), *hs:sox2/hs:sox2* (P, S) and *hs:sox3/hs:sox3* (Q, T) embryos following heat-shock (39°C, 60 minutes) at 24 hpf. All images show dorsolateral views (anterior to the left) and otic vesicle borders are outlined.

Moderate misexpression of *sox2* or *sox3* at 24 hpf had no discernable effect on subsequent sensory or neurogenic domains (data not shown), but high-level misexpression at 24 hpf strongly suppressed expression of *atoh1a* and *neurog1* by 26 hpf (Fig. 3.4I–K, O–Q). By 30 hpf, expression of *atoh1a* and *neurog1* recovered to near normal in *hs:sox2/hs:sox2* embryos (Fig. 3.4M, S), whereas *hs:sox3/hs:sox3* embryos continued to show partial suppression of *atoh1a* and *neurog1* (Fig. 3.4N, T). Thus, the ability of *sox2* and *sox3* to expand sensory and neurogenic domains is gradually lost during later stages of otic development and instead high-level misexpression strongly suppresses sensory and neural development. This suggests that *sox2* and *sox3* promote an early state of sensory and neural competence while delaying early fate-specification, similar to their roles in establishing the neural plate during gastrulation (Archer et al., 2011; Bylund et al., 2003; Okuda et al., 2010; Rogers et al., 2009).

**A delayed response to early misexpression of *sox2* and *sox3***

To better characterize the effects of early misexpression, we examined when changes in *atoh1a* and *neurog1* expression first become evident following high-level misexpression of *sox2* and *sox3*. In *hs:sox2/hs:sox2* embryos heat shocked at 12.5 hpf, expression of *atoh1a* was normal through 16.5 hpf (Fig. 3.5B), with the first signs of moderate expansion appearing by 18.5 hpf (Fig. 3.5E). Expression of *neurog1* was reduced in *hs:sox2/hs:sox2* at 16.5 hpf but showed

moderate expansion by 18.5 hpf (Fig. 3.5H, K). In *hs:sox3/hs:sox3* embryos heat shocked at 12.5 hpf, the domain of *atoh1a* was partially expanded at 16.5 hpf and continued to expand through 18.5 hpf (Fig. 3.5C, F). High-level misexpression of *sox3* did not accelerate the onset of *neurog1* expression, but the *neurog1* domain was already partially expanded by 16.5 hpf and continued to expand through 18.5 hpf (Fig. 3.5I, L). To test whether expansion of sensory and neurogenic domains involved elevated proliferation, we examined patterns of phospho-histone H3 staining after misexpressing *sox2* or *sox3* at 12.5 hpf. There was no significant change in the number of phospho-Histone H3 positive cells in the otic vesicle in *hs:sox2/hs:sox2* or *hs:sox3/hs:sox3* embryos at any point before or during sensory-neural expansion (Fig. 3.5M, N). Thus, early activation of *sox2* or *sox3* did not immediately or directly induce sensory and neural fates, nor promote cell proliferation to expand the sensory and neurogenic domains. Instead, the delayed response to misexpression suggests that transient elevation of *sox2* or *sox3* during placodal stages causes lasting changes in developmental programming that enhance competence to form sensory and neural fates in the otic vesicle.



**Figure 3.5. *sox2* and *sox3* do not directly specify neural or sensory fates.**

(A-F) Dorsolateral views (anterior to the left) showing otic expression of *atoh1a* at 16.5 hpf (A-C) and 18.5 hpf (D-F) in control (A, D), *hs:sox2/hs:sox2* (B, E) and *hs:sox3/hs:sox3* (C, F) embryos following heat-shock at 12.5 hpf. (G-L) Dorsolateral views showing otic expression of *neurog1* at 16.5 hpf (G-I) and 18.5 hpf (J-L) in control (G, J), *hs:sox2/hs:sox2* (H, K) and *hs:sox3/hs:sox3* (I, L) embryos following heat-shock at 12.5 hpf. Otic vesicle borders are outlined in all images. (M, N) Quantification of the number of phospho-Histone H3 positive (PH3+) cells in the otic vesicle of control and *hs:sox2/hs:sox2* (M) or *hs:sox3/hs:sox3* (N) embryos at multiple time points following heat-shock at 12.5 hpf. Error bars represent standard deviation. No statistically significant differences between control and *hs:sox2/hs:sox2* or

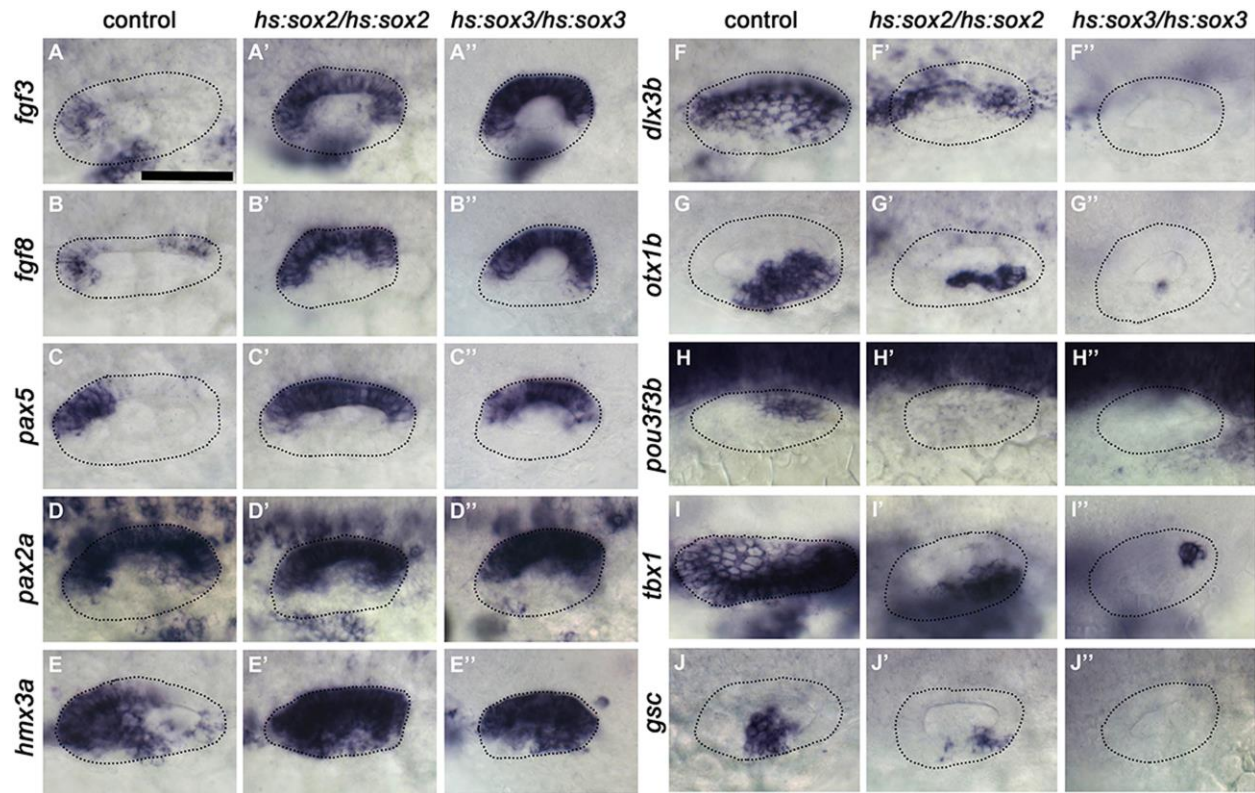


**Figure 3.5. Continued.**

*hs:sox3/hs:sox3* embryos at any time point examined (student's t-test,  $n > 15$ ). In all panels, *hs:sox2/hs:sox2* embryos were heat shocked at 39°C for 60 minutes, whereas *hs:sox3/hs:sox3* embryos were heat shocked at 38°C for 30 minutes.

**Effects of misexpression of *sox2* and *sox3* on patterning in the otic vesicle**

The strong expansion of sensory and neurogenic domains following high-level misexpression of *sox2* or *sox3* suggested dramatic changes in patterning of the otic vesicle. To test this, we analyzed the expression of a variety of regional markers at 24 hpf after high-level misexpression of *sox2* or *sox3* at 12.5 hpf. Anterior markers *fgf3* and *pax5* were expanded throughout the medial wall of the otic vesicle at 24 hpf, as was *fgf8* (Fig. 3.6A–C''), and the anterior-ventral marker *hmx3a* expanded to include nearly all cells in otic vesicle (Fig. 3.6E–E''). Medial expression of *pax2a* was not altered (Fig. 3.6D–D'') but the posterior-medial marker *pou3f3b* was lost in all embryos (Fig. 3.6H–H''). Posterior-lateral markers *otx1b* and *gsc* and the dorsal marker *dlx3b* were strongly reduced in *hs:sox2/hs:sox2* embryos and were eliminated in *hs:sox3/hs:sox3* embryos (Fig. 3.6F–F'', G–G'', J–J''). Similarly, *tbx1*, a marker of non-neural and non-sensory fates, was nearly eliminated in all embryos (Fig. 3.6I–I''). These data show that early misexpression of *sox2* and *sox3* leads to expansion of anterior-ventral identity in almost all cells in the otic vesicle, corresponding to the region shared by utricular sensory and neural fates. Despite this dramatic change in axial patterning, most embryos maintained a clear sensory-neural boundary, corresponding to the lateral boundary of the *pax2a* domain (see below).



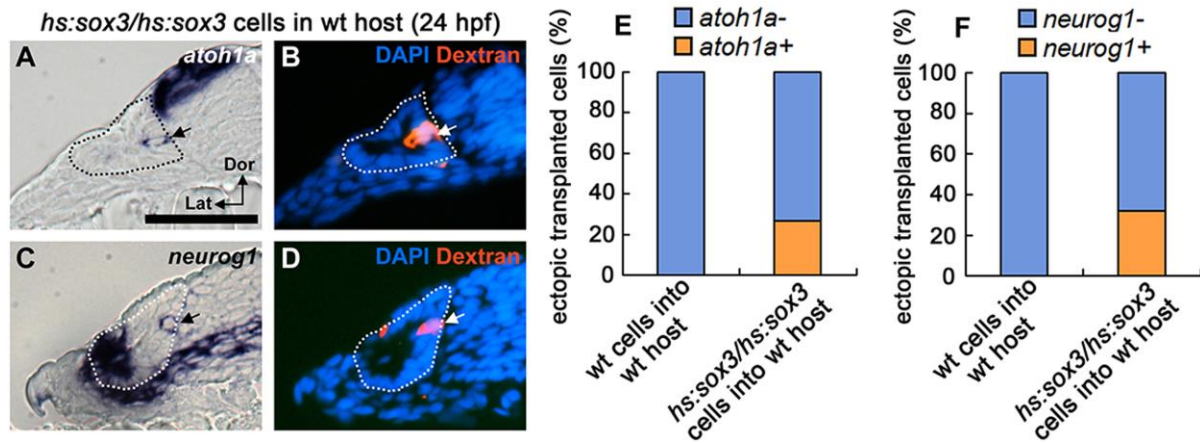
**Figure 3.6. Axial patterning in the otic vesicle following early high-level misexpression of *sox2* or *sox3*.**

Dorsolateral views (anterior to the left) showing expression of various regional markers at 24 hpf in the otic vesicle of control (A-J), *hs:sox2/hs:sox2* (A'-J') and *hs:sox3/hs:sox3* (A''-J'') embryos following heat-shock at 12.5 hpf. *hs:sox2/hs:sox2* embryos were heat shocked at 39°C for 60 minutes, *hs:sox3/hs:sox3* embryos were heat shocked at 38°C for 30 minutes. Otic vesicle borders are outlined in all images.

### Mosaic misexpression of *sox3*

Because global misexpression could potentially alter surrounding tissues that normally provide signals needed for proper patterning of the otic vesicle, we transplanted *hs:sox3/hs:sox3* cells into wild-type host embryos to test the effects of mosaic misexpression. We reasoned that if early misexpression of *sox3* acts cell-autonomously to enhance sensory-neural competence, then transplanted *hs:sox3/hs:sox3* cells should be able to adopt sensory and neural fates in ectopic

locations within the otic vesicle. In support, when mosaic embryos were heat shocked at 12.5 hpf, 26.7% of transplanted *hs:sox3/hs:sox3* cells located in regions outside endogenous sensory epithelia expressed *atoh1a* ectopically at 24 hpf (n = 32 embryos, 105 transplanted cells) (Fig. 3.7A, B, E), whereas no wild-type cells transplanted into wild-type host embryos expressed *atoh1a* ectopically (n = 8 embryos, 63 ectopic transplanted cells) (Fig. 3.7E). Similarly, 31.9% of transplanted *hs:sox3/hs:sox3* cells located outside the endogenous neurogenic domain expressed *neurog1* ectopically at 24 hpf (n = 9 embryos, 72 transplanted cells) (Fig. 3.7C, D, F), whereas no control transplants expressed *neurog1* ectopically (n = 11 embryos, 83 ectopic transplanted cells) (Fig. 3.7F). These data support the idea that elevating *sox3* at 12.5 hpf cell-autonomously enhances pro-sensory and pro-neural competence of otic cells.

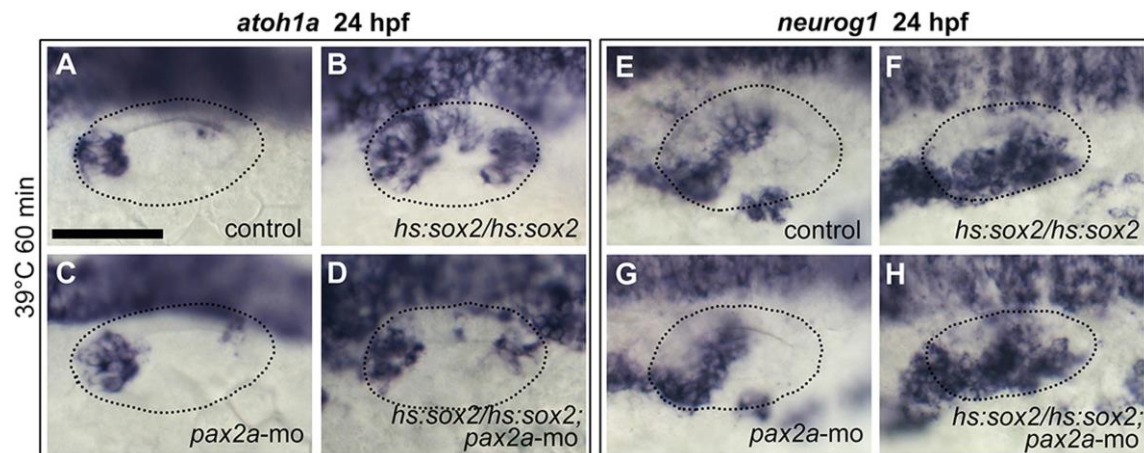


**Figure 3.7. Effects of early misexpression of *sox3* in genetic mosaics.**

(A-D) Expression of *atoh1a* (A) and *neurog1* (C) at 24 hpf in cross sections of wild-type hosts into which fluorescent dextran-labeled *hs:sox3/hs:sox3* transgenic cells were transplanted. Mosaic embryos were heat shocked at 12.5 hpf, 38°C for 30 minutes. (B, D) Fluorescent image of dextran and DAPI staining on the same sample shown in A and C respectively. Sections pass through the middle of the otic vesicle, just posterior to the utricular macula. Arrows indicate transgenic cells that ectopically express *atoh1a* or *neurog1*. Otic vesicle borders are outlined. (E, F) Quantification of the percentage of transgenic cells located outside endogenous sensory or neural domains that ectopically express *atoh1a* (E) or *neurog1* (F).

### Interaction with *pax2a* influences *sox2* and *sox3* function

Previous studies suggested that SoxB1 factors can physically or genetically interact with other transcription factors to modify their functions (Ambrosetti et al., 1997, Boer et al., 2007, Chew et al., 2005, Kamachi et al., 2001, Kondoh and Kamachi, 2010). Because *pax2a* expression is restricted to the medial wall of the otic vesicle and helps regulate sensory development (Riley et al., 1999), we hypothesized that Pax2a locally biases the activity of Sox2 and Sox3 to promote sensory fate. To test this, we examined the effects of misexpressing *sox2* or *sox3* in *pax2a*<sup>-/-</sup> mutants or *pax2a* morphants. Disruption of *pax2a* function did not block formation of endogenous sensory epithelia (Fig. 3.8C; and Riley et al., 1999) but suppressed the ability of early high-level misexpression of *sox2* to expand the sensory domain of *atoh1a* at 24 hpf (compare Fig. 3.8B, D). Importantly, knocking down *pax2a* did not suppress the ability of *sox2* to expand the neurogenic domain of *neurog1* (Fig. 3.8F, H). Similarly, disruption of *pax2a* suppressed the ability of *sox3* misexpression to expand the sensory domain of *atoh1a* whereas expansion of the neurogenic domain of *neurog1* still occurred (data not shown). These data support the hypothesis that the pro-sensory effect of Sox2 and Sox3 requires Pax2a, whereas the pro-neural function does not.



**Figure 3.8. Pax2a is required for prosensory but not proneural expansion.**

**Figure 3.8. Continued.**

(A-H) Dorsolateral views (anterior to the left) showing otic expression of *atoh1a* (A-D) and *neurog1* (E-H) at 24 hpf in control embryos (A, E), *pax2a*-morphants (C, G), *hs:sox2/hs:sox2* homozygotes (B, F) and *hs:sox2/hs:sox2* homozygotes injected with *pax2a*-mo (D, H). Embryos were heat shocked at 12.5 hpf, 39°C for 60 minutes. Otic vesicle borders are outlined in all images.

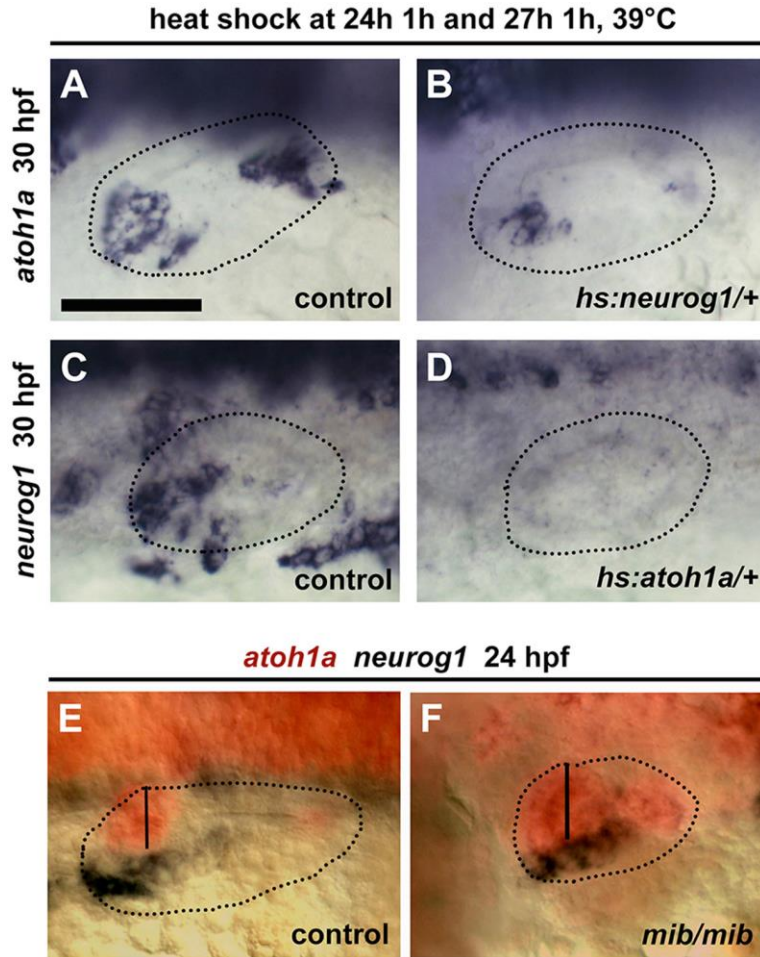
**Mutual repression between *atoh1a* and *neurog1***

In mouse embryos, neurogenesis and sensory development occur sequentially from the same spatial domain. The transition from neurogenesis to sensory development is regulated in part by mutual repression between *Neurog1* and *Atoh1* (Raft et al., 2007). To test whether a similar cross-repression helps reinforce or maintain the sensory-neural boundary in zebrafish, we activated *hs:atoh1a/+* or *hs:neurog1/+* at 24 hpf when sensory and neurogenic domains are already well established and then examined subsequent effects on *neurog1* or *atoh1a* expression at 30 hpf. We previously reported that serial activation of *hs:atoh1a* by heat shocking embryos 24 hpf and 27 hpf provides optimal expansion of sensory epithelia (Sweet et al., 2011). Under these same conditions, serial activation of *hs:atoh1a* eliminated expression of *neurog1* at 30 hpf (Fig. 3.9C, D). Conversely, serial activation of *hs:neurog1* at 24 hpf and 27 hpf strongly repressed expression of *atoh1a* at 30 hpf (Fig. 3.9A, B). These data support the idea that the mutual antagonism between *atoh1a* and *neurog1* helps maintain the sensory-neural boundary during otic development in zebrafish.

Because *Neurog1-Atoh1* cross-repression in mouse is mediated in part by Notch signaling (Raft et al., 2007), we examined whether disruption of Notch signaling in *mind bomb* (*mib*) mutants alters the sensory-neural boundary in zebrafish. Two-color in situ hybridization showed that *mib*



mutants exhibit strong upregulation of both *atoh1a* and *neurog1*, but the *atoh1a-neurog1* boundary is nevertheless maintained (Fig. 3.9E, F). This indicates that Notch activity represses expression of both *atoh1a* and *neurog1* but is not required for spatial segregation of sensory and neural fates in zebrafish.

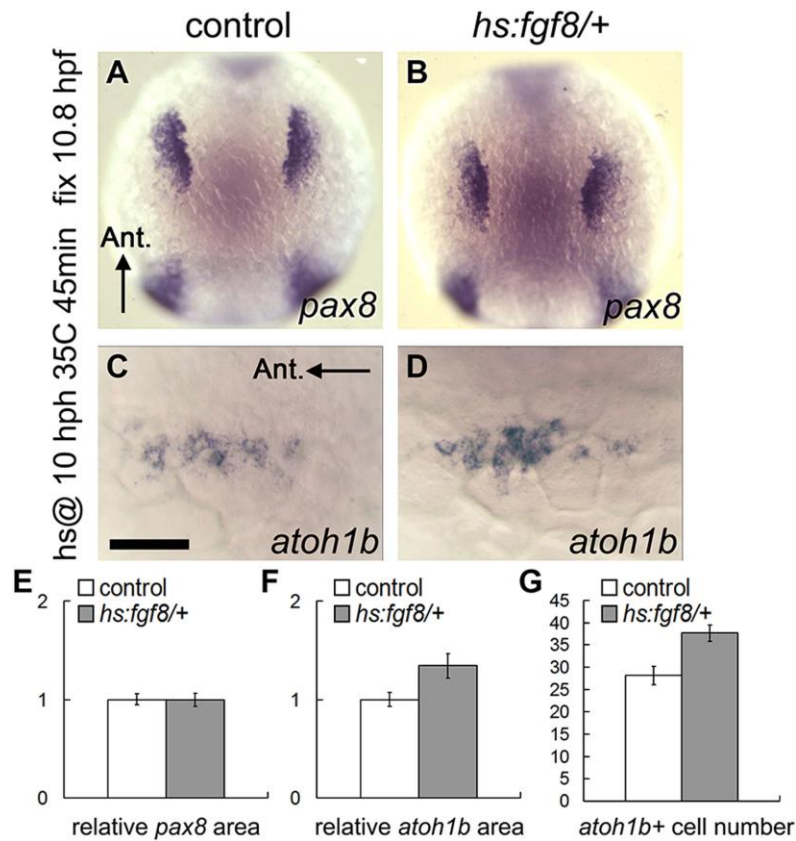


**Figure 3.9. Roles for *atoh1a-neurog1* cross-repression but not Notch in sensory-neural segregation.**

(A, B) Expression of *atoh1a* at 30 hpf in a control (A) and *hs:neurog1/+* (B) embryo following serial heat shock at 24 and 27 hpf, for 60 min at 39°C. (C, D) Expression of *neurog1* at 30 hpf in a control (C) and *hs:atoh1a/+* (D) embryo following serial heat shock at 24 and 27 hpf, for 60 min at 39°C. (E, F) Co-staining of *atoh1a* (red) and *neurog1* (black) expression by two-color in situ hybridization in a control embryo (E) and a *mib* homozygote mutant (F) at 24 hpf. Otic vesicle borders are outlined in all images. The width of the utricular macula is marked by vertical lines. All images show dorsolateral views with anterior to the left.

### Early expansion of sensory potential by Fgf

The earliest sign of sensory development occurs at roughly 10.5 hpf (tail bud stage) when *atoh1b* is induced at the medial edge of the otic placode (Millimaki et al., 2007), less than one hour after Fgf-dependent induction of *pax8* in the nascent otic anlagen (Phillips et al., 2001). Expression of *atoh1b* is later required for timely activation of *sox2* in the sensory domain (Millimaki et al., 2010). We showed previously that maintaining expression of *atoh1b* requires Fgf (Millimaki et al., 2007), but this is difficult to interpret because blocking Fgf at 10 hpf destabilizes otic development (Léger and Brand, 2002). Additionally, high-level misexpression of Fgf at 10 hpf enlarges the entire otic placode (Padanad et al., 2012), including the early domain of *atoh1b* expression. We therefore tested whether misexpressing Fgf at a low level could expand the domain of *atoh1b* without increasing the number of *pax8*-expressing otic cells. For this experiment, *hs:fgf8/+* heterozygotes were subjected to a very mild heat shock at 35 °C beginning at 10 hpf and embryos were fixed at 10.8 hpf to examine expression of *atoh1b*. Under these conditions, the size of the *pax8* domain was not altered (Fig. 3.10A, B, E) but the domain of *atoh1b* expression expanded by 34% based on measuring spatial area or by counting *atoh1b*<sup>+</sup> cells (Fig. 3.10C, D, F, G). The enlarged domain of *atoh1b* was not maintained after the heat shock, as the number of *atoh1b*<sup>+</sup> cells declined to normal by 14 hpf (not shown) in a process previously shown to involve Notch-dependent domain-restriction (Millimaki et al., 2007). Nevertheless, these data indicate that the level of Fgf signaling can influence the proportion of pre-otic cells able to express prosensory markers.



**Figure 3.10. Low-level misexpression of Fgf8 expands sensory potential in the early placode.**

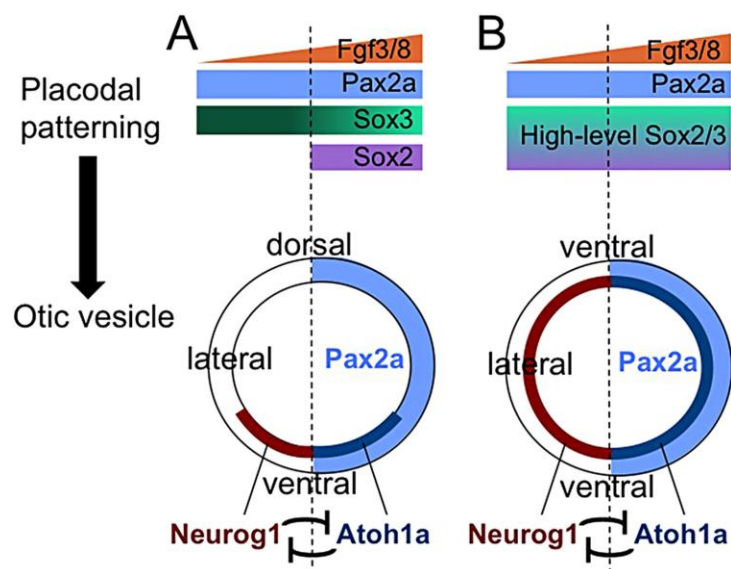
(A-D) Dorsal views showing expression of *pax8* (A, B, anterior up) and *atoh1b* (C, D, anterior to the left) at 10.8 hpf in control (A, C) and *hs:fgf8/+* (B, D) embryos that were heat shocked at 10 hpf, 35°C for 45 minutes. (E-G) Quantification of relative surface area of otic/epibranchial *pax8* domain (E), *atoh1b* domain (F) and the number of *atoh1b*-expressing cells (G) in control and *hs:fgf8/+* embryos following heat shock at 10 hpf, 35°C for 45 minutes. Error bars represent standard error of the mean. Asterisks indicate statistically significant differences compared to control (\*P<0.05, student's t-test, n>22).

## DISCUSSION

Our findings support a model in which *sox2* and *sox3* provide unique functions during placodal development to establish sensory and neurogenic competence, respectively (Fig. 3.11A). Previous studies suggest that SoxB1 functions can vary based on intrinsic differences in protein



structure, expression level, or availability of cofactors. It appears that all three variables influence *sox2* and *sox3* functions during otic development in zebrafish. First, several observations suggest that the functions of *sox2* and *sox3* are intrinsically different. Although expression of *sox2* and *sox3* overlap in the prosensory region, mutant analysis shows that only *sox2* is required for normal sensory development, and double mutants show no further impairment. Additionally, moderate misexpression of *sox2* expands sensory domain while reducing the neurogenic domain. Second, the functions of both genes depend on their level of expression. Specifically, *sox2* and *sox3* can mimic each other when misexpressed at high levels, leading to dramatic expansion of the sensory and neurogenic domains in the otic vesicle (Fig. 3.11B). Third, regionally expressed cofactors appear to modify the functions of Sox2 and Sox3: The medial factor *pax2a* is required to expand the sensory domain following misexpression of either *sox2* or *sox3*, whereas the neurogenic domain is unaffected (Fig. 3.11A, B). Thus the overlap of multiple mechanisms allows otherwise similar SoxB1 factors to perform distinct functions in abutting domains of the otic vesicle.



**Figure 3.11. Summary and model of sensory-neural patterning.**

(A) Under normal conditions, a gradient of Fgf from the mesendoderm and hindbrain induces the formation of otic placode, in which *pax2a* is uniformly expressed, *sox3* is initially uniformly expressed but by 12 hpf is reduced in medial cells, and *sox2* is restricted to medial cells. Sensory

**Figure 3.11. Continued.**

competence is favored by the overlap between *sox2*, *pax2a*, elevated Fgf, and reduced *sox3*. Neurogenic competence is favored by elevated *sox3*, the absence of *sox2*, and moderate Fgf. These early patterns help establish spatial segregation of sensory and neurogenic domains in the floor of the otic vesicle, in which cross-repression between Neurog1 and Atoh1a helps reinforce or maintain segregation. (B) When Sox2/3 is transiently overexpressed at 12.5 hpf, prosensory and proneural competence increases in all otic cells. Later, sensory and neurogenic domains expand throughout the otic vesicle. This is accompanied by loss of non-sensory-neural fates and expansion of anterior-ventral identity throughout the otic vesicle, the region normally shared by utricular sensory and neural fates. Despite these changes in axial patterning, medial-lateral segregation of sensory and neural domains, and the medial domain of Pax2a, are maintained.

The ability of *sox2* and *sox3* misexpression to dramatically expand sensory and neurogenic domains correlates with global expansion of the anterior-ventral markers of the otic vesicle (Fig. 3.6). This phenotype is remarkable in several ways. First, regional fates in the otic vesicle normally rely on inductive interactions from surrounding tissues. However, early misexpression of *sox2* or *sox3* stably specifies anterior-ventral identity. That is, cells are not respecified by signals that normally establish distinct regional identities. Although global misexpression of *sox2/3* potentially alters such signals, genetic mosaics containing isolated transgenic cells also show a high incidence of sensory or neural development in ectopic locations. With such sparse distribution of transgenic cells it is unlikely that signaling interactions from surrounding tissues are significantly altered, suggesting that sensory and neural competence persists regardless of changing regional signals. We speculate that *sox2* and *sox3* function in the otic vesicle much as they do in the early neural plate, wherein SoxB1 factors stably specify a zone of neurogenic potential while simultaneously preventing premature neural differentiation. Subsequently, rising levels of neurogenic bHLH transcription factors repress expression of SoxB1 factors as cells begin to differentiate. Such a transition is seen in the neurogenic domain of the otic vesicle, wherein *sox3* is expressed in a gradient with levels declining towards the lateral edge where neuroblasts delaminate (Fig. 3.1E;

Kantarci et al., 2016). The transition is also evident in sensory epithelia when hair cells upregulate *atoh1a* while losing expression of *sox2* (Millimaki et al., 2010).

The second notable feature of the phenotype caused by early misexpression of *sox2* or *sox3* is that the medial-lateral segregation of sensory and neurogenic fates is maintained and continues to respect the *pax2a* expression boundary. Although *pax2a* expression is initially expressed throughout the otic placode, it overlaps with the medial/prosensory domain of *sox2* as early as 12 hpf (6 somites, Gou et al., 2018). As the otic vesicle forms, expression of *pax2a* becomes restricted to the medial wall but continues to overlap with *sox2* in the ventromedial quadrant. Expression of *pax2a* does not depend on *sox2* but is nevertheless required for expansion of sensory epithelia by *sox2/3* misexpression. Thus the *sox2-pax2a* partnership defines the prosensory compartment throughout early otic development. In contrast, the ability of *sox3* to promote neural competence appears to require the absence of *sox2* (Fig. 3.2G), whereas *pax2a* is superfluous. Neurogenic competence neither requires *pax2a* nor is it impaired by misexpression of *pax2a* (Kantarci et al., 2016).

### **Basis for unique functions of Sox2 and Sox3**

Although most studies conclude that Sox2 and Sox3 functions are largely redundant, there are several examples in which these proteins exhibit distinct functions. For instance, human embryonic stem cells (hESCs) express both *Sox2* and *Sox3*, but their functions are not identical. Either factor is sufficient to maintain pluripotency, but *Sox2* alone can promote hESC self-renewal whereas *Sox3* cannot (Wang et al., 2012). In neural progenitors, too, some functions of *Sox2* and *Sox3* are non-redundant as each factor activates a different set of neural makers (Archer et al.,

2011; Rogers et al., 2009; Rogers et al., 2014). The mechanistic basis for such differences is unknown but possibly reflects structural differences in the transactivation domain that alter the ability to interact with different cofactors (Cox et al., 2010, Kondoh and Kamachi, 2010). Additionally, Sox2 and Sox3 can show markedly different affinities for specific DNA sequences (Collignon et al., 1996), indicating that small changes in their HMG DNA-binding domains also facilitate distinct functions.

### **Comparison with chick and mouse**

In chick, *Sox2* and *Sox3* show overlapping expression during otic development. Expression of *Sox3* begins during early placodal development, marking the nascent neurogenic domain and promoting subsequent expression of *Neurog1* (Abello et al., 2010). Expression of *Sox2* begins later and is retained in sensory epithelia, whereas *Sox3* expression is lost after the neural-sensory transition (Neves et al., 2007). These data support a functional bias for *Sox3* in neurogenic competence and *Sox2* for sensory competence, similar to what we found in zebrafish. Overexpression of *Sox2* in chick can induce *Neurog1* (Evsen et al., 2013), though under these conditions it may mimic the normal function of *Sox3* as we have found in zebrafish.

In mouse, *Sox2* is expressed in the otic vesicle and is required for both neural and sensory development (Kiernan et al., 2005, Puligilla et al., 2010, Steevens et al., 2017), whereas *Sox3* expression is not detected. How *Sox2* alone mediates both functions is not understood, but comparison with zebrafish suggests several possibilities. First, the level of *Sox2* expression could be sufficiently high in mouse that it can fulfill both functions, similar to our misexpression studies in zebrafish. Second, functional output could be influenced by interactions with *Pax2*. The early

neurogenic domain in mouse straddles the *Pax2* expression boundary (Burton et al., 2004), and it is likely that most neuroblasts arise from the lateral (*Pax2*-negative) domain. The lateral neurogenic domain overlaps a ventrolateral domain of *Gooseoid* (Vitelli et al., 2003), which in zebrafish is required for neuroblasts to delaminate and is repressed by *pax2a* (Kantarci et al., 2016). In mouse, vestibular and auditory neurons do not detectably express *Pax2* (Lawoko-Kerali et al., 2002), and loss of *Pax2* does not block formation of vestibular or spiral ganglia (Burton et al., 2004). In contrast, *Pax2* is abundantly expressed in sensory epithelia, especially in differentiating hair cells (Lawoko-Kerali et al., 2002), and loss of *Pax2* perturbs development of sensory epithelia (Burton et al., 2004; Zou et al., 2006). It is possible that Pax2 physically interacts with Sox2 to activate sensory-specific enhancers, analogous to Pax6-Sox2 activation of lens-specific enhancers (Kamachi et al., 2001). Alternatively, Pax2 and Sox2 can also bind independently to widely separated binding sites within specific enhancers to drive expression in sensory epithelia (Robert-Moreno et al., 2011).

### **Relative roles Neurog1-Atoh1a cross-repression and Notch**

The transition from neural to sensory development in birds and mammals is triggered in part by *Neurog1*-dependent activation of Notch (Brooker et al., 2006; Daudet et al., 2007) (Daudet and Lewis, 2005; Neves et al., 2011), as well as cross-repression between *Neurog1* and *Atoh1* (Raft et al., 2007). In zebrafish, too, *atoh1a* and *neurog1* show cross-repression which presumably helps maintain and sharpen the sensory-neural border. However, Notch activity plays no role in spatial segregation between these domains in zebrafish, since a sharp boundary persists in *mib* mutants. Rather Notch acts independently in both domains to limit *atoh1a* and *neurog1* activity. This function is critical for establishing the alternating pattern of hair cells and support cells in sensory

epithelia (Haddon et al., 1998, Millimaki et al., 2007, Riley et al., 1999) and to limit the pace of neuroblast specification and differentiation (Kantarci et al., 2015).

### **The role of Fgf**

The earliest sign of prosensory development in zebrafish is the induction of *atoh1b* in the medial portion of the otic placode (Millimaki et al., 2007). Activation of *atoh1b* requires Fgf signaling and is limited to medial cells in close proximity to the hindbrain-source of Fgf. We showed that low-level activation of *hs:fgf8* is sufficient to increase the number of *atoh1b*-expressing cells without increasing the total number of *pax8*-positive otic cells (Fig. 3.10). The early otic domain of *atoh1b* does not reflect overt sensory specification since the domain is later restricted to only a few prospective hair cells by Notch-dependent lateral inhibition (Millimaki et al., 2007). However, the early *atoh1b* domain can nevertheless be viewed as an early marker of prosensory competence or potential, since knockdown of *atoh1b* blocks differentiation of the first hair cells and delays expression of *atoh1a* by many hours (Millimaki et al., 2007, Millimaki et al., 2010). While the above data suggest that elevating Fgf expands prosensory competence, it is unclear whether there is a corresponding contraction of neurogenic competence. However, we showed previously that weak activation of *hs:fgf8* does cause downregulation of *sox3* to a discrete lower level in the otic placode (Bhat and Riley, 2011; Padanad and Riley, 2011). Whether this molecular change reflects reduced neurogenic competence remains to be determined.

## CHAPTER IV

### SUMMARY AND ADDITIONAL DISCUSSIONS

#### SUMMARY OF FINDINGS

Sox2 and Sox3 are closely related SoxB1 family transcription factors that play important functions in neural development, especially the central nervous system, which is the focus of many previous studies. This dissertation focuses on elucidating their roles in development of sensory organ and peripheral nervous system, specifically the inner ear and sensory neuron of the epibranchial system in zebrafish.

The study in Chapter II examined the role of *sox2* and *sox3* during the induction of otic and epibranchial placodes, the precursors of inner ear and epibranchial ganglia. Here we discovered that low-level expression of *sox2* can be detected in the otic/epibranchial region as early as 12–12.5 hpf, two hours earlier than previously reported. To test the involvement of *sox2* and *sox3* in early placodal development, we generated knockout lines and heat shock-inducible transgenic lines for *sox2* and *sox3*. Phenotypes of *sox2* or *sox3* null mutants closely mimic phenotypes of their respective morphants. Mutant analysis confirms that *sox2* and *sox3* provide partially redundant functions required for establishing a normal amount of otic and epibranchial tissue. Consistent with functional redundancy, low-level misexpression of *sox2* can rescue placodal deficiencies in *sox3*<sup>-/-</sup> mutants, and deficiency in epibranchial ganglia is significantly more severe in double mutants for *sox2* and *sox3* than in single mutants. Although both factors are required for proper

placode induction, there appears to be an upper limit for expression level of *sox2* and *sox3* during pro-placodal development. High-level misexpression of either *sox2* or *sox3* cell-autonomously blocks initial otic induction, but does not block or reverse the fate of otic cells once they have begun to differentiate. Moreover, in an assay to induce ectopic otic tissue, the ability of Fgf misexpression to induce ectopic *pax8* is enhanced by partial knockdown of *sox3*, but is inhibited by full knockdown of *sox3*. We also re-examined the genetic interaction between *sox3* and *pax8*, using double mutant line for *sox3* and *pax8* generated in this study, to validate a previously reported morphant phenotype. We confirmed that *pax8*<sup>-/-</sup> mutants (like *pax8*-morphants) show a more severe placodal deficiency than *sox3*<sup>-/-</sup> mutants, but the placodal deficiency is no worse in *pax8*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants. This suggests that *sox3* and *pax8* work together in the same pathway rather than providing distinct gene-specific functions. Together these results in Chapter II show that *sox2* and *sox3* are required at an optimal level to ensure proper induction of otic and epibranchial placodes, either higher or lower levels hinders this process, and they are partially redundant at this stage.

The study in Chapter III investigated the functions of *sox2* and *sox3* during later stages of otic development using knockout lines and heat shock-inducible transgenes. Our mutant analysis showed that *sox2* is uniquely required for normal sensory development while *sox3* is uniquely required for neural development in zebrafish inner ear. In support of their unique roles, misexpression of *sox2* at a moderate level during placodal stage results in expansion of sensory domains (*atoh1a*<sup>+</sup>) and reduction of the neurogenic domain (*neurog1*<sup>+</sup>) later in the otic vesicle. However, high-level misexpression of *sox2* or *sox3* causes them to mimic each other's function, dramatically expanding domains of sensory and neurogenic fates to fill the medial and lateral halves of the otic vesicle, respectively. Analysis of axial markers shows that this *sox2* or *sox3*



misexpression expands anterior-ventral identity (including utricular sensory and neural fates) throughout the otic vesicle, at the expense of posterior-dorsal identity. Interaction with medial factor *pax2a* is required for *sox2* or *sox3* misexpression to be able to expand sensory but not neurogenic domains. Misexpression of *sox2* or *sox3* at placodal stage did not accelerate the onset of prosensory and proneural factors *atoh1a* and *neurog1*, nor promote proliferation of otic cells, rather expansion of sensory and neural fates happened gradually as the otic vesicle formed. *sox2* and *sox3* misexpression at later stages in the otic vesicle suppressed expression of *atoh1a* and *neurog1* temporarily. Additionally, mild misexpression of *fgf8* during placodal development was sufficient to expand the zone of prosensory competence specifically. Together, these data in Chapter III show that *sox2* and *sox3* exhibit intrinsic differences in promoting sensory vs. neural competence, but at high levels these factors can mimic each other to enhance both states. We also propose a novel model (Fig. 3.10 Chapter III) where regional cofactors like *pax2a* and *fgf8* also help *sox2/3* to establish sensory and neural fates in separate domains, whose boundary is later reinforced by cross-repression between *atoh1a* and *neurog1*.

## **FUNCTION OF EARLY PLACODAL EXPRESSION OF SOX2**

We found that low-level expression of *sox2* can be detected in the otic/epibranchial region as early as 12–12.5 hpf. Loss of *sox2* results in about 15% reduction of otic/epibranchial domain marked by *pax2a* at 12 hpf, although the domain size is normal at 11 hpf. These data uncovered the involvement of *sox2*, in addition to *sox3*, in establishing a normal size of otic/epibranchial domain. Previous studies suggested that the process of otic/epibranchial placode induction has three general phases: the initial otic induction phase (9-11hpf), secondary otic recruitment phase

(11-12.5 hpf) and epibranchial induction phase (11.5-13 hpf) (Bhat and Riley, 2011; Maulding et al., 2014; Padanad and Riley, 2011). During the secondary otic recruitment phase, cells surrounding the otic region join the growing placode by directed migration, a convergence process that requires *integrin-a5* (*itga5*) (Bhat and Riley, 2011). Once they migrate into the range of otic inducing Fgf signals, they start to express *pax8/pax2a* (Bhat and Riley, 2011). Because *sox2* does not express in the otic/epibranchial domain until 12 hpf, and loss of *sox2* does not alter the domain size at 11 hpf, it is highly unlikely that it contributes to otic induction phase. Rather, the timing of otic/epibranchial *sox2* expression, and loss-of-function phenotype at 12 hpf, suggests that *sox2* is involved in secondary otic recruitment phase. There are two possibilities: (1) loss of *sox2* could affect *itga5* expression, which would lead to reduction of cells that migrate into otic region; or, (2) *sox2* could help maintaining the fate of newly recruited otic cells when they migrated into the otic region and were induced to express *pax2a/pax8*. However, *itga5* morphants have much more pronounced deficiency of otic tissue at 24 hpf (Bhat and Riley, 2011) than *sox2*<sup>-/-</sup>, which recovered to normal size (Fig. 1.1 Chapter II), making the first possibility less likely. Although future studies are needed to fully rule out the first hypothesis, general functions of SoxB1 factors presented in Chapter II argue in favor of the second possibility.

## THE ROLE OF SOX2 AND SOX3 DURING EPIBRANCHIAL DEVELOPMENT

We showed that *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants have a significantly greater deficiency of epibranchial ganglia than single mutants. Although, it is worth noting that *sox2*<sup>-/-</sup> mutants have a slight decrease in epibranchial ganglia. This is a much milder deficiency compared to *sox3*<sup>-/-</sup> mutants, which have a strong decrease in all epibranchial ganglia, especially in facial ganglion

(80% reduction). This is consistent with the difference in expression pattern between *sox2* and *sox3*: *sox2* is weakly expressed in the early otic/epibranchial region at 12 hpf, but later quickly restrained to prosensory domains of the otic placode by 13-14 hpf (Millimaki et al 2010), and are not detectable in epibranchial domain anymore; whereas expression of *sox3* defines the developing epibranchial placodes/ganglion until at least 26 hpf (Nikaido et al., 2007; Sun et al., 2007). Therefore, although *sox2* and *sox3* are both required for establishing normal amount of epibranchial precursors, *sox3* is more critical, and potentially could be involved in multiple steps of epibranchial development in addition to its role in epibranchial induction. For example, *sox3* could play a role during later epibranchial neurogenesis, like its role in promoting otic neurogenesis (Chapter III). Conditional knockout of *sox3* is needed to examine its late role without affecting epibranchial induction.

## **REDUNDANCY AND COMPENSATION BETWEEN SOX2 AND SOX3 DURING EPIBRANCHIAL DEVELOPMENT**

Removing *sox2* in *sox3*<sup>-/-</sup> mutants lead to additional loss of epibranchial tissue, especially in facial and glossopharyngeal ganglion. This could be the result of loss of compensation from *sox2*, as the 2-fold increase of *sox2* transcript abundance observed in *sox3*<sup>-/-</sup> mutants (Fig. 2.1 Chapter II) could be ameliorating the effect of losing *sox3*. Nonetheless, this 2-fold increase of *sox2* expression is not sufficient to rescue early epibranchial defects in *sox3*<sup>-/-</sup> mutants. It is possible that higher level of *sox2* or earlier misexpression of *sox2* is needed. Whether *sox2* can fully substitute *sox3* during epibranchial development remains to be tested, by knocking *sox2* into *sox3* coding region and swapping out *sox3*.

## EARLY REQUIREMENT OF SOX2 AND SOX3 IN SEQUENTIAL INDUCTION OF EPIBRANCHIAL PLACODE

Previous published work suggests that induction of epibranchial placodes happens in an anterior to posterior sequence: facial placode induction requires Fgf signaling strongest around 11.5 hpf, while requirement for Fgf to induce glossopharyngeal and vagal placodes happens after 11.5 hpf (Nechiporuk et al., 2007). In our study, the facial ganglion is the most deficient (over 80% reduction) among all epibranchial ganglia in *sox3*<sup>-/-</sup> mutants and *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants, followed by glossopharyngeal ganglion which shows stronger reduction than vagal ganglion in *sox2*<sup>-/-</sup>; *sox3*<sup>-/-</sup> double mutants (Fig. 2.4. Chapter III). These findings suggest that function of *sox2* and *sox3* is especially critical around 11.5 hpf, when facial and glossopharyngeal placodes are being induced sequentially. This coincide with the timing of early placodal expression of *sox2* and *sox3*, as at 11.5 hpf *sox3* is the sole SoxB1 factor expressed in otic/epibranchial domain helping induction of facial precursors, while *sox2* starts to express at 12 hpf to help *sox3* in induction of glossopharyngeal precursors. Later forming vagal ganglia are less affected by loss of *sox2* and *sox3*, suggesting other factors might act redundantly during development of vagal ganglia.

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